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Improved Sensitivity Mass Spectrometric Detection of Eicosanoids by Charge Reversal Derivatization

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Combined liquid chromatography−electrospray ionization-tandem mass spectrometry (LC−ESI-MS/MS) is a powerful method for the analysis of oxygenated metabolites of polyunsaturated fatty acids including eicosanoids. Here we describe the synthesis of a new derivatization reagent *N*-(4-aminomethylphenyl)pyridinium (AMPP) that can be coupled to eicosanoids via an amide linkage in quantitative yield. Conversion of the carboxylic acid of eicosanoids to a cationic AMPP amide improves sensitivity of detection by 10- to 20-fold compared to negative mode electrospray ionization detection of underivatized analytes. This charge reversal derivatization allows detection of cations rather than anions in the electrospray ionization mass spectrometer, which enhances sensitivity. Another factor is that AMPP amides undergo considerable collision-induced dissociation in the analyte portion rather than exclusively in the cationic tag portion, which allows isobaric derivatives to be distinguished by tandem mass spectrometry, and this further enhances sensitivity and specificity. This simple derivatization method allows prostaglandins, thromboxane B_2, leukotriene B_4, hydroxyeicosatetraenoic acid isomers, and arachidonic acid to be quantified in complex biological samples with limits of quantification in the 200−900 fg range. One can anticipate that the AMPP derivatization method can be extended to other carboxylic acid analytes for enhanced sensitivity detection.

Liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC−ESI-MS/MS) has emerged as a powerful method to detect oxygenated derivatives of fatty acids including eicosanoids (for example, refs 1−3). With these methods it is possible to analyze a large collection of eicosanoids in a single LC−ESI-MS/MS run. These lipid mediators are detected by single reaction monitoring (SRM) in which precursor ions are isolated in the first stage of the mass spectrometer followed by collision-induced dissociation to give fragment ions, which are detected after an additional stage of mass spectrometer isolation. The current limit of quantification for these analytes is in the ∼10−20 pg range. This sensitivity level is appropriate for studies with cultured cells *in vitro* or with relatively large tissue samples, but it is not sufficient for studies with smaller volume samples such as joint synovial fluid or bronchoalveolar lavage fluid from experimental rodents. Given the importance of oxygenated fatty acid derivatives in numerous medically important processes such as inflammation and resolution of inflammation, we sought to improve the LC−ESI-MS/MS sensitivity of detection of these lipid mediators using a widely available analytical platform.

For reasons that are not well understood, cations generally form gaseous ions better than anions in the electrospray ionization source of the mass spectrometer. Additionally, for underivatized carboxylic acids it is required to add a weak organic acid to the chromatographic mobile phase, i.e., formic acid, so that the carboxylic acid is kept in its protonated state, which allows it to be retained on the reverse-phase column to ensure chromatographic separation. However, the presence of the weak acid offsets the formation of carboxylate anions in the electrospray source because the weak acid carries most of the anionic charge in the electrospray droplets, and thus formation of analyte anions is suppressed. We reasoned that conversion of the carboxylic acid to a fixed-charge cationic derivative would lead to improved detection sensitivity by ESI-MS/MS. Charge-reversal derivatization of carboxylic acids with quaternary amines has been explored in previous work (for example, refs 4−6). However, these reagents utilize organic cations that tend to fragment by collision-induced dissociation near the cationic site. Fragmentation in the derivatization tag is not desirable because analytes that form isobaric precursor ions and that comigrate on the LC column will not be distinguished in the mass spectrometer if they give rise to the same detected fragment ion. This loss of analytical specificity is a serious problem when analyzing complex biological samples. Fragmentation in the analyte portion rather than in the tag portion

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also reduces chemical noise, which also enhances sensitivity of detection.

In this study we report the design and synthesis of a new cationic tag and show that it can be quantitatively attached via an amide linkage to the carboxyl group of eicosanoids by a simple derivatization procedure. We then show that the derivatized eicosanoids can be analyzed by LC−ESI-MS/MS with limits of quantification that are well below those reported for underivatized eicosanoids.

EXPERIMENTAL METHODS

Synthesis of AMPP. Pyridine (40 mmol, 3.2 mL) was dissolved in 46 mL of absolute ethanol followed by the addition of 1-chloro-2,4-dinitrobenzene (40 mmol, 8.2 g, Aldrich). The mixture was heated with a reflux condenser at 98 °C for 16 h under nitrogen. After cooling, ethanol was removed by rotary evaporation, and the crude product was recrystallized by dissolving in a minimal amount of hot ethanol and allowing the solution to slowly cool. The product *N*-2,4 dinitrophenyl pyridinium chloride was isolated as a yellow solid in 62% yield, and its identity was confirmed by melting point analysis (189−191 °C observed, 189−190 °C reported[7]). *N*-2,4-Dinitrophenyl pyridinium chloride (16.8 mmol, 4.76 g) was dissolved in 70 mL of ethanol−pyridine (3:1). 4-[(*N*-Boc) -amino-methyl] aniline (33.6 mmol, 7.56 g, Aldrich) was added, and the reaction mixture was heated under a reflux condenser at 98 °C under nitrogen for 3 h. After cooling, 700 mL of water was added to precipitate 2,4-dinitroaniline. After filtration, the filtrate was concentrated to dryness by rotary evaporation, and the product was isolated as a brown oil. This oil was treated with 112 mL of 25% (v/v) trifluoroacetic acid in dichloromethane for 30 min at room temperature. The mixture was concentrated by rotary evaporation, and the solid was triturated twice with benzene to remove excess trifluoroacetic acid. The mixture was again concentrated by rotary evaporation. The residue was dissolved in a minimal amount of heated ethanol, the solution was allowed to cool for ∼5 min, and then diethyl ether was added with swirling until the solution started to cloud up. The mixture was transferred to the freezer (−20 °C) and left overnight. The mixture was allowed to warm to room temperature and then decanted. The obtained mother liquor was treated with additional diethyl ether as above to give additional AMPP solid. The solids were combined and triturated with diethyl ether. The solid was dried under vacuum to give 3.30 g of AMPP as a brown solid, 59% yield. ^{1}H NMR (300 MHz, D_6-DMSO) 9.34 (d, 2H), 8.81 (t, 1H), 8.53 (broad, 3H), 8.33 (t, 2H), 7.95 (d, 2H), 7.80 (d,2H), 4.22 (s, 2H) (^{1}H NMR spectra are shown in the Supporting Information, estimated purity of AMPP is >95%).

Preparation of Eicosanoid Stock Solutions. The following eicosanoid standards from Cayman Chemicals were used (PGE_2, PGD_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, TxB_2, 5(*S*)-HETE, 8(*S*)-HETE, 11(*S*)-HETE, 12(*S*)-HETE, 15(*S*)-HETE, LTB_4, arachidonic acid, D_4-PGE_2, D_4-PGD_2, D_4-$PGF_{2\alpha}$, D_4-6-keto-$PGF_{1\alpha}$, D_4-TXB_2, D_8-5(*S*)-HETE, D_4-LTB_4, D_8-arachidonic acid). Stock solutions of eicosanoids were prepared at a concentration of 100 pg/μL in absolute ethanol and stored at −80 °C under Ar in Teflon septum, screw cap vials. Serial dilutions of the stock solutions were made in absolute ethanol for standard curve and extraction analysis. Internal standards were diluted to a working stock of 5 pg/μL in absolute ethanol.

Preparation of Samples Prior to Derivatization with AMPP. *Standard Curves.* Each sample contained 50 pg of each internal standard and various amounts of nonisotopic eicosanoids (added from the stock solutions described above) transferred to a glass autosampler vial insert (Agilent catalog no. 5183-2085). Solvent was removed with a stream of nitrogen, and the residue was derivatized with AMPP as described below.

Mouse Serum. Analysis of endogenous eicosanoids in serum was carried out with commercial mouse serum (Atlantic Biologicals catalog no. S18110). A volume of 1, 5, or 10 μL of serum was placed in a glass autosampler vial insert. Two volumes of methanol (LC/MS, JT Baker catalog no. 9863-01) containing 50 pg of each internal standard were added. The vial insert was mixed on a vortex mixer for ∼10 s. The concentration of methanol was lowered to 10% (v/v) by addition of purified water (Milli-Q, Millipore Corp.), and the samples were loaded via a glass Pasteur pipet onto a solid phase extraction cartridge (10 mg Oasis-HLB, Waters catalog no. 186000383). The cartridges were previously washed with 1 mL of methanol and then 2 × 0.75 mL of 95:5 water−methanol. After sample loading, the sample tube was rinsed with 200 μL of purified water−methanol (95:5, v/v), and this was added to the cartridge. The cartridge was washed with 2 × 1 mL of water−methanol (95:5, v/v). Additional solvent was forced out of the cartridge solid phase by applying medium pressure N_2 (house N_2 passed through a 0.2 μm cartridge filter) for a few seconds. Column eluant receiver vials (Waters Total Recovery autosampler vials, Waters catalog no. 186002805) were placed under the cartridges. The cartridges were then eluted with methanol (1 mL). All cartridge steps were carried out using a vacuum manifold (Waters catalog no. WAT200606) attached to a water aspirator. Solvent was removed by placing the receiver vials in a centrifugal evaporator (Speed-Vac). These processed samples were derivatized with AMPP (see below) without storage.

Lung Epithelial Cells. The University of Washington Institutional Review Board approved the studies involving human subjects, and written informed consent was obtained from all participants. Primary bronchial epithelial cells were isolated from a volunteer with asthma during a bronchoscopy using a nylon cytology brush of cells from subsegmental airways. To establish primary culture, the epithelial cells were seeded into a culture vessel coated with type 1 collagen in bronchial epithelial basal media (BEBM, Lonza, Allendale, NJ) supplemented with bovine pituitary extract, insulin, hydrocortisone, gentamicin, amphotericin B, fluconazole, retinoic acid, transferrin, triiodothyronine, epinephrine, and human recombinant epidermal growth factor (serum-free BEGM) and maintained at 37 °C in a humidified incubator. After expansion *in vitro*, passage 2 epithelial cells were grown to >90% confluence on a 12-well plate. The medium was changed to 200 μL of Hanks balanced salt solution, and the cells were treated with either calcium ionophore (A23187, 10 μM in DMSO) or a DMSO-containing control solution for 20 min at 37 °C. The synthesis of eicosanoids was stopped by the addition of 4 volumes of ice-cold methanol with 0.2% formic acid, and samples were stored at −80 °C until processed. The number of epithelial

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cells was 5.0×10^5 cells per well. The studies were performed in duplicate.

Frozen samples were thawed on ice, 50 pg of each internal standard was added, and Milli-Q water was added to bring the methanol to 10% (v/v). The samples were processed by solid-phase extraction (Oasis-HLB) as described for mouse serum samples.

Rat 3Y1 Cells. 3Y1 cells were a gift from Dr. H. Kuwata (Department of Health Chemistry, School of Pharmaceutical Sciences, Showa University). 3Y1 cells were maintained in complete medium consisting of Dulbecco's Modified Eagle Medium (DMEM) with low glucose (Invitrogen catalog no. 11885-084) containing 10% heat inactivated, fetal bovine serum (FBS) (Invitrogen catalog no. 16140), 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen catalog no. 15140) in plastic tissue culture dishes (Nunc catalog no. 172958) in a humidified atmosphere of 5% CO_2 at 37 °C. Passage of cells was performed using 0.25% trypsin/EDTA (Invitrogen catalog no. 25200-056).

For PGE_2 analysis, 3Y1 cells were plated at 5×10^4 cells/well in a 24-well plate (Nunc catalog no. 142475) in 1 mL of complete medium and incubated overnight. The medium was then replaced with DMEM containing 2% FBS. After 24 h incubation, the medium was removed from each well and replaced with 1 mL DMEM containing 2% FBS and the cytosolic phospholipase A_2-α inhibitor Wyeth-2 (compound 10 from ref 9) or DMSO vehicle control (final concentration in each well did not exceed 0.1% (v/v)). Cells were incubated for 20 min at room temperature. Mouse interleukin-1β (1 ng/well) and human tumor necrosis factor-α (1 ng/well) (R & D Systems catalog no. 401-ML and 210-TA) were both added to each well in 10 μL of DMEM containing 2% FBS (negative control wells received only 10 μL of DMEM containing 2% FBS). Cells were incubated for 48 h at 37 °C in a humidified atmosphere of 5% CO_2. The supernatant was then carefully removed from each well and transferred into a glass test tube and placed on ice for immediate analysis. PGE_2 levels were measured by an enzyme immunoassay kit (Cayman Chemical catalog no. 500141) according to the manufacturer's instructions using 50 μL of medium above the cells. A sample of medium (50 μL) was also analyzed by LC−ESI-MS/MS as follows. To the medium was added 2 volumes of methanol containing 50 pg of each internal standard, methanol was reduced to 10% v/v by addition of Milli-Q water, and samples were submitted to solid-phase extraction using Oasis-HLB cartridges as described for mouse serum samples.

Derivatization with AMPP. To the residue in the Waters Total Recovery autosampler vial was added 10 μL of ice-cold acetonitrile (JT Baker catalog no. 9017-03)−*N*,*N*-dimethylformamide (Sigma catalog no. 227056) (4:1, v:v). Then 10 μL of ice-cold 640 mM (3-(dimethylamino)propyl)ethyl carbodiimide hydrochloride (TCI America catalog no. D1601) in purified water was added. The vial was briefly mixed on a vortex mixer and placed on ice while other samples were processed as above. To each vial was added 20 μL of 5 mM *N*-hydroxybenzotriazole (Pierce catalog no. 24460)−15 mM AMPP in acetonitrile. The vials were mixed briefly on a vortex mixer, capped, and placed in a 60 °C incubator for 30 min. The cap was replaced with a split-septum screw cap (Agilent catalog no. 5185-5824) for autoinjection onto the LC−ESI-MS/MS. Samples were analyzed on the same day. Samples were kept in the autosampler rack at 4 °C while queued for injection.

LC−ESI-MS/MS Analysis. Some studies were carried out with a Waters Quattro Micro triple quadrupole mass spectrometer, a 2795 Alliance HT LC/autosampler system, and the QuanLynx software package. Chromatography was carried out with a C18 reverse-phase column (Ascentis Express C18, 2.1 mm × 150 mm, 2.7 μm, Supelco catalog no. 53825-U). Solvent A is 95% H_2O/5% CH_3CN/1% acetic acid, and solvent B is CH_3CN/1% acetic acid. The solvent program is (linear gradients) 0−1.0 min, 95−78% A; 1.0−7.0 min, 78−74% A; 7.0−7.1 min, 74−55% A; 7.1−12.1 min, 55−40% A; 12.1−13.0 min, 40−0% A; 13.0−15.0 min, 0% A; 15.0−15.1 min, 0−95% A; 15.1−20.1 min, 95% A. The flow rate is 0.25 mL/min.

Similarly, some studies were carried out with a Waters Quattro Premier triple quadrupole mass spectrometer interfaced to an Acquity UPLC. Solvent A is 100% water (Fisher Optima grade catalog no. L-13780)−0.1% formic acid (Fluka catalog no. 94318), and solvent B is CH_3CN (Fisher Optima grade catalog no. L-14338)−0.1% formic acid. The same LC column was used but with a modified solvent program (linear gradients): 0−1.0 min, 95% A; 1.0−2.0 min, 95%−85% A; 2.0−2.1 min, 85%−74% A; 2.1−6.0 min, 74%−71% A; 6.0−6.1 min, 71%−56% A; 6.1−10.0 min, 56% A; 10.0−14.0 min, 56%−0% A; 14.0−14.1 min, 0%−95% A; 14.1−18.0 min, 95% A. Supplemental Material Tables 1 and 2 in the Supporting Information give the autosampler and ESI-MS/MS data collection parameters for the Waters Quattro Micro triple quadrupole and the Waters Quattro Premier triple quadrupole mass spectrometers, respectively.

For comparison purposes, we also carried out LC−ESI-MS/MS analysis of underivatized eicosanoids in the negative ion mode. The same LC column, solvents A and B, and flow rate were used as for the AMPP amides. The solvent program was slightly modified as follows: For the Waters Quattro Micro, we used 0−1.0 min, 95−63% A; 1.0−7.0 min, 63%A; 7.0−7.1 min, 63−38% A; 7.1−12.1 min, 38−23% A; 12.1−13.0 min, 23−0% A; 13.0−15.0 min, 0% A; 15.0−15.1 min, 0−95% A; 15.1−20.1 min, 95% A. For the Waters Quattro Premier, we used 0−1.0 min, 95% A; 1.0−2.0 min, 95%−85% A; 2.0−2.1 min, 85%−59% A; 2.1−6.0 min, 59% A; 6.0−6.1 min, 59%−38% A; 6.1−11 min, 38%−23% A; 11−11.1 min, 23%−0% A; 11.1−14 min, 0% A; 14.0−14.1 min, 0%−95% A; 14.1−19.0 min, 95% A. Values of m/z for precursor and fragment ions were as published,[8] and cone voltages and collision energies were optimized for each instrument and for each analyte in the usual way (values not shown but similar to those published[8]).

Eicosanoid Recovery Studies. We measured the recovery of eicosanoids following the sample workup procedure given above. Recovery studies were done using either 30 mg Strata-X (Phenomenex Cat. 8B−S100-TAK-S) or 10 mg Oasis-HLB (Waters Cat. 186000383) cartridges with 50 or 5 pg of each eicosanoid in phosphate buffered saline. For these recovery studies, 50 pg of each internal standard was added to samples just prior to derivatization with AMPP (i.e., post-solid phase extraction).

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Table 1. Extraction Yields, Liquid Chromatography Retention Times, and Tandem Mass Spectrometry Parameters for Eicosanoid AMPP Amide Molecular Species

eicosanoid	extraction yield (%)[a]	LC retention time (min)[b]	limit of quantitation in positive mode (pg) (CV)[c]	limit of quantitation in negative mode (pg)[d]	precursorion[e] (m/z)	fragment ion[e] (m/z)	cone voltage[f] (V)	collision energy[f] (eV)
6-keto-$PGF_{1\alpha}$	102/87	5.4/4.1	5/0.3 (6.8)	110/10	537	239	65/80	55/55
$PGF_{2\alpha}$	64/100	6.7/5.2	5/0.5 (9.1)	110/7	521	239	60/75	43/52
PGE_2	67/74	6.8/5.3	5/0.3 (5.5)	100/7	519	239	60/75	40/45
PGD_2	63/67	7.2/5.7	5/0.6 (7.5)	120/7	519	307	60/75	40/48
TxB_2	86/86	6.2/4.6	5/0.2 (2.3)	90/4	537	337	65/70	43/42
LTB_4	42/63	9.8/7.7	5/0.5 (10.3)	80/7	503	323	50/60	35/35
5(*S*)-HETE	42/65	10.4/9.7	5/0.4 (10.4)	120/10	487	283	55/65	37/34
8(*S*)-HETE	40/75	10.3/9.1	5/0.2 (6.7)	120/10	487	295	55/65	37/40
11(*S*)-HETE	31/66	10.2/8.8	5/0.3 (8.9)	80/5	487	335	55/65	32/30
12(*S*)-HETE	69/62	10.2/8.9	10/0.9 (6.6)	140/10	487	347	55/65	35/35
15(*S*)-HETE	35/53	10.1/8.5	5/0.4 (11.1)	200/10	487	387	55/65	33/34
arachidonic acid	73/113	11.7/12.4	10/1 (5.6)	no data	471	239	55/65	40/50
D_4-6-keto-$PGF_{1\alpha}$		5.4/4.1			541	241	65/80	55/55
D_4-$PGF_{2\alpha}$		6.7/5.2			525	241	60/75	43/52
D_4-PGE_2		6.8/5.3			523	241	60/75	40/45
D_4-PGD_2		7.1/5.6			523	311	60/75	40/48
D_4-TxB_2		6.2/4.6			541	341	65/70	43/42
D_4-LTB_4		9.8/7.7			507	325	50/60	35/35
D_8-5(*S*)-HETE		10.4/9.6			495	284	55/65	37/34
D_8-arachidonic acid		11.7/12.4			479	239	55/65	40/50

[a] The first number is for the Waters Oasis HLB cartridge, and the second number is for the Phenomenex Strata-X cartridge. [b] The first number is for the Waters Quattro Micro, and the second number is for the Waters Quattro Premier. [c] LOQ values are for eicosanoid AMPP amides in positive mode. The first number is for the Waters Quattro Micro, and the second number is for the Waters Quattro Premier. The values in parentheses are the CVs based on 6 independent 15 μL injections of 0.075 pg/μL on the Waters Quattro Premier (see the main text). [d] LOQ values are for underivatized eicosanoids analyzed by LC−ESI-MS/MS in negative mode. The first number is for the Waters Quattro Micro, and the second number is for the Waters Quattro Premier. [e] m/z values listed are calculated monoisotopic values. The actual values used are derived from instrument tuning, which is instrument dependent. [f] Cone voltages and collision energies were optimized for each analyte. These numbers are instrument dependent. The first number is for the Waters Quattro Micro instrument, and the second number is for the Waters Quattro Premier instrument.

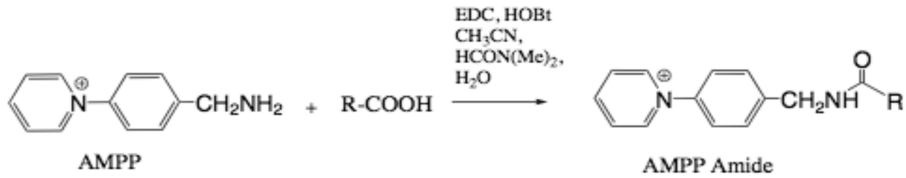

Figure 1. Structure of AMPP and an AMPP amide along with the reagents used for derivatization.

Recoveries were obtained by comparing the LC−ESI-MS/MS peak integrals to those obtained from a sample of eicosanoids that were derivatized with AMPP and injected directly onto the LC column without sample processing. Recovery yields are given in Table 1. The peak areas for the internal standards were similar in all samples studied (not shown) showing that the eicosanoid recoveries were similar regardless of sample matrix.

RESULTS AND DISCUSSION

Design and Preparation of AMPP Amides. Our goal was to develop a simple derivatization procedure to convert the carboxylic acid terminus of eicosanoids to a cationic group so that mass spectrometry could be carried out in positive mode. The tag should be designed so that fragmentation occurs in the analyte portion rather than in the tag portion so that the power of tandem mass spectrometry can be used for analytical specificity and sensitivity. Our computational analysis of substituted *N*-pyridinium methylamino derivatives[10] indicated that these had only moderate dissociation energies for loss of pyridine and other single bond cleavages in the linker and thus might not be suitable for our purposes, which require fragmentation in the fatty acyl chain. Therefore, the charge tag was redesigned to strengthen the pyridinium N−C bond by inserting a phenyl ring as a linker. The phenyl ring also serves to enhance the derivative's interaction with the LC reverse phase column, which is necessarily used to minimize any matrix effects during ESI-MS/MS analysis. Density functional theory calculations (B3LYP/6-31G*) indicated that homolytic cleavage of the benzylic CH_2−N bond in the charge tag was a high-energy process that required >350 kJ mol^{-1} of dissociation energy and was deemed not to out compete fragmentations in the fatty acyl chain (Supplementary Material Figure 1 in the Supporting Information). A side reaction that could not be evaluated with the model system shown in Supplementary Material Figure 1 in the Supporting Information is elimination of 4-(*N*-pyridyl)benzylamine, which gives rise to the m/z 183 marker fragment ion. An amide linkage of the charge tag to the analytic carboxylic acid was preferred over an ester since the former are generally more resistant to fragmentation in the mass spectrometer.[11] The final feature of the design of AMPP is its ease of synthesis in pure form.

We developed a simple synthesis of highly pure *N*-(4-aminomethylphenyl)-pyridinium (AMPP, Figure 1) and used it as a new derivatization reagent. We developed a simple method to convert

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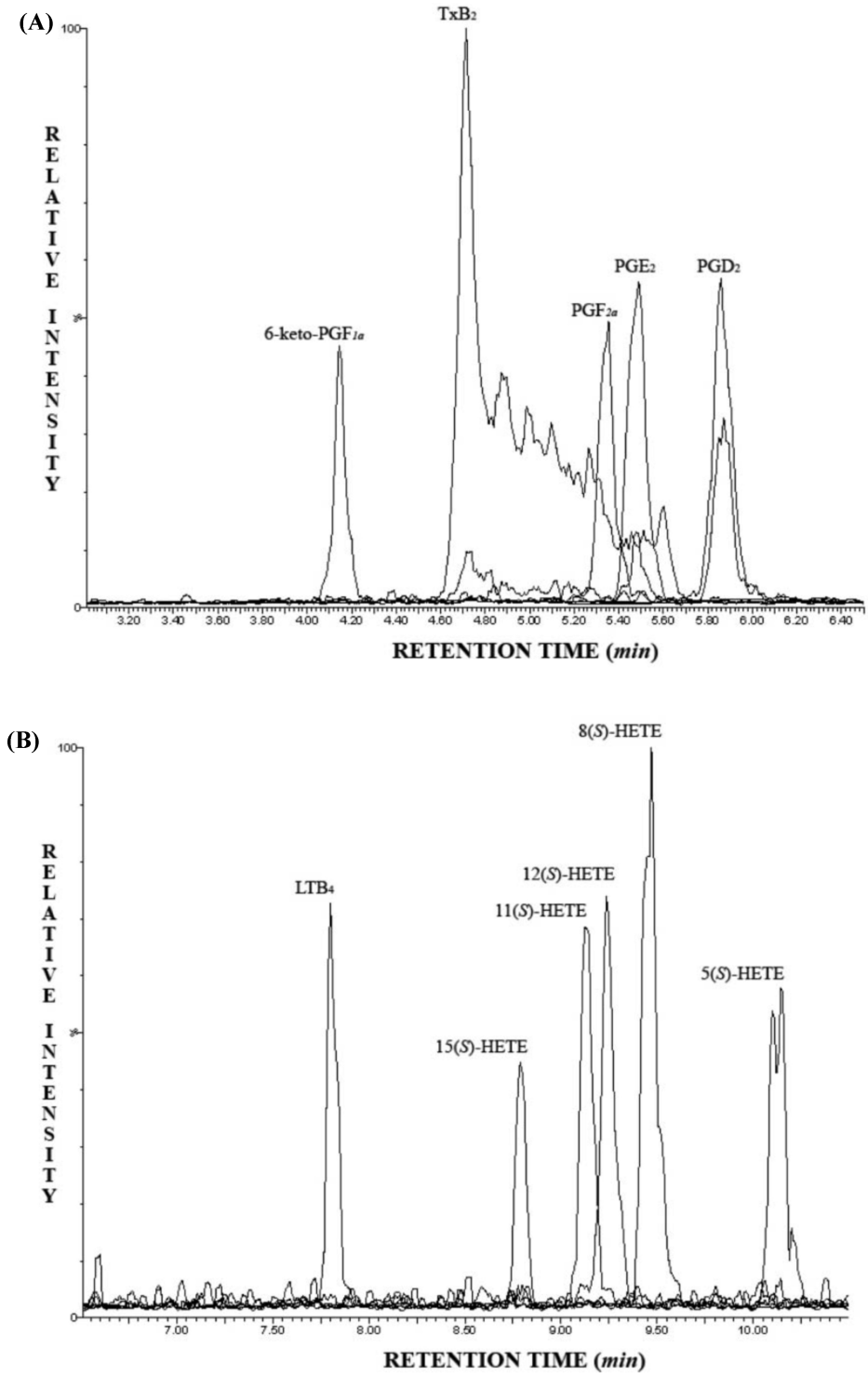

Figure 2. LC−ESI-MS/MS traces of eicosanoids after workup from a solution in phosphate buffered saline: (A) early eluting eicosanoids and (B) latter eluting eicosanoids.

the carboxyl group of lipid mediators to the AMPP amide using a well-known carbodiimide coupling reagent. Coupling conditions were optimized to give near quantitative formation of the AMPP amide. The was shown by converting the fluorescent fatty acid 10-pyrene-decanoic acid into its AMPP amide and examining the reaction mixture by fluorimetric HPLC and ESI-MS. No remaining 10-pyrenedecanoic acid was detected, and the AMPP amide was formed in >95% yield with <1% *N*-acyl urea formation (a common side product in carbodiimide-promoted couplings) (data not shown). Derivatization is carried out using a single reagent cocktail in a small vial heated at 60 °C for 30 min without the need for anhydrous or anaerobic conditions.

LC−ESI-MS/MS Analysis of Eicosanoid AMPP Amides. In this study we focused on representative eicosanoids that do not contain peptides such as cysteinyl leukotrienes. The analytes studied are PGD_2, PGE_2 and $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ (the spontaneous breakdown product from prostacyclin, PGI_2), TxB_2 (the spontaneous breakdown product from TxA_2), LTB_4, 5(*S*)-HETE), 8(*S*)-HETE, 11(*S*)-HETE, 12(*S*)-HETE, and 15(*S*)-HETE, and AA. ESI-MS/MS analysis with fragment ion

scanning of AMPP amides of the analytes are shown in the Supporting Information Supplementary Material Figure 6A−D. In all the spectra, some cleavage of the AMPP tag occurs giving rise to the peaks at $m/z = 169$ and 183. To best ensure analytical specificity, we quantified the eicosanoids using a fragment in the analyte portion rather than in the AMPP tag. For PGD_2, we used the $m/z = 307$ fragment, which is likely due to cross-ring cleavage of the cyclopentanone ring and represents the most abundant non-tag fragment ion. For PGE_2 and $PGF_{2\alpha}$, we used $m/z = 239$ due to cleavage between C3 and C4. For AA and 6-keto-$PGF_{1\alpha}$, we used $m/z = 239$, again due to C3−C4 cleavage. The major nontag fragment for TxB_2 is $m/z = 337$ due to cross-ring cleavage. For all HETE species, we could identify a unique fragment ion (see Table 1) to avoid cross contamination of MS/MS signals due to partially unresolved LC peaks. Finally, for LTB_4, we chose $m/z = 323$, which is the most abundant non-tag fragment ion but has a nonobvious origin.

Next we studied a mixture of eicosanoids in phosphate buffered saline and optimized pre-ESI-MS/MS sample workup and LC−ESI-MS/MS conditions. Critical for a successful method with ultrasensitive analyte detection is high yield recovery of analytes from the sample prior to LC−ESI-MS/MS analysis. We found that solid phase extraction with a rapidly wettable matrix (Oasis HLB cartridges, Waters Inc. or Strata-X cartridges, Phenomenex) combined with analyte elution with methanol gave high yield recovery of all eicosanoids analyzed (Table 1). The data in Table 1 is based on 50 pg of each analyte submitted to recovery studies. When the same recovery studies were carried out with 5 pg of eicosanoid, results were essentially identical (data not shown). Inferior recoveries were obtained using elution of the solid phase cartridges with acidified methanol or if the sample was subjected to liquid−liquid extraction or protein precipitation using various solvents (Supporting Information). Figure 2A,B shows a typical LC−ESI-MS/MS run of an eicosanoid mixture. All of the analytes examined elute from the LC column in less than 11 min. All HETEs except 11(*S*)-HETE and 12(*S*)-HETE are baseline resolved. The partial overlap of the 11(*S*)- and 12(*S*)-HETEs is not a concern because completely selective fragment ions are being monitored. The LC elution profile and peak shape for both the derivatized and underivatized analytes (not shown) are similar. This shows that the presence of the quaternary ammonium group of the AMPP tag has no negative effect on the LC. TxB_2 gives rise to the typical broad peak shape due to interconversion between the two hemiacetal isomers. Also similar to underivatized analysis, the fragments used to monitor PGD_2 and PGE_2 (isobars of each other) are not completely unique to each species, but baseline LC resolution of these two eicosanoids resolves the issue. The same is true for the isobaric pair TxB_2 and 6-keto-$PGF_{1\alpha}$.

To ensure high yield conversion of eicosanoids to AMPP amides with minimal formation of *N*-acyl-ureas, we used a large excess of derivatization reagents. The AMPP tag and the EDCI coupling reagent elute in the void volume (not shown), and the HOBt elutes in the large time window between PGD_2 and LTB_4 (not shown). Thus removal of excess derivatization reagents is not necessary prior to LC−ESI-MS/MS.

Standard curve analysis was performed starting below the limit of quantification up to 2 orders of magnitude above this limit. In all cases, the mass spectrometry response was linear from the limit of quantification up to the highest amount analyzed (19 pg on-column for the Waters Quattro Micro and 5−10 pg on-column for the Waters Quattro Premier) (Supplemental Material Figure 2A,B in the Supporting Information). We define limit of quantification as the amount of eicosanoid AMPP amide needed to give an ESI-MS/MS signal that is 10-fold the noise (as determined using the Waters MassLynx software). The limits of quantification for all eicosanoid AMPP amides are listed in Table 1. The values using the Waters Quattro Micro are 5 pg for all eicosanoids except 12(*S*)-HETE (10 pg) and AA (10 pg). With the Waters Quattro Premier, the values are 0.2−0.5 pg for all eicosanoids except 12(*S*)-HETE (0.9 pg) and AA (1 pg). We also determined limit of quantification of underivatized eicosanoids analyzed by LC−ESI-MS/MS in negative ion mode. The values are shown in Table 1 for the Waters Quattro Micro and the Waters Quattro Premier. The new method using AMPP amides is found to be 10- to 20-fold more sensitive on both instruments. This constitutes a significant improvement in detection sensitivity of eicosanoids.

To validate the above stated estimates of the limit of quantification, we injected eicosanoids standard mixtures six times on the Waters Quattro Premier, where each analyte was injected at roughly twice the estimated limit of quantification. Values of CV for analyte to internal standard peak ratios for each analyte are listed in Table 1. These CVs validate our estimates for the limits of quantification by demonstrating that eicosanoid analysis can be performed with reasonable reproducibility proximal to these stated values.

Next, we more thoroughly investigated the reproducibility of the AMPP derivatization method for eicosanoid analysis. The results are summarized in Table 2. Three repetitive LC−ESI-MS/MS analyses of an identical sample (19 pg on-column levels of each eicosanoid) gave coefficients of variation in the range of the 2.4−12.7% range. We also prepared three independent mixtures of eicosanoids (50 pg each) in phosphate buffered saline, submitted each to sample workup, and analyzed each by LC−ESI-MS/

Table 2. Coefficient of Variation (%) for LC−ESI-MS/MS Analysis of Eicosanoid AMPP Amides

	intrasample[a]	intersample (10 μL PBS)[b]	intersample (10 μL serum)[c]
6-keto-$PGF_{1\alpha}$	4.9	16.9	not detected
TxB_2	2.4	3.0	3.7
$PGF_{2\alpha}$	8.1	6.5	6.4
PGE_2	8.0	9.2	not detected
PGD_2	4.5	8.3	not detected
LTB_4	7.3	17.2	7.3
5(*S*)-HETE	3.3	2.8	6.4
8(*S*)-HETE	6.7	20.3	13.7
11(*S*)-HETE	5.5	12.2	8.8
12(*S*)-HETE	5.2	10.7	6.3
15(*S*)-HETE	4.0	5.8	12.4
arachidonic acid	12.7	28.2	7.9

[a] Coefficient of variation for the analysis of the same sample of 1.25 pg/μL of eicosanoid standards injected three times onto the LC−ESI-MS/MS. [b] Coefficient of variation for the analysis of three independent samples of 1.25 pg/μL of eicosanoid standards (50 pg of standards spiked into phosphate buffered saline and worked up as described in the Experimental Methods section for LC−ESI-MS/MS.). [c] Coefficient of variation for three separate serum samples spiked with internal standards only.

Table 3. Eicosanoid Levels (pg/μL) in Mouse Serum and Bronchial Epithelial Cells[a]

serum (μL)	TxB_2	PGE_2	PGD_2	$PGF_{2(}$	LTB_4	5(*S*)-HETE	8(*S*)-HETE	11(*S*)-HETE	12(*S*)-HETE	15(*S*)-HETE	Arachi-donic Acid
1	46			15	198	748	393	437	11 345	240	28 623
1	49			11	340	1075	545	646	17 840	703	23 246
5	43			13	174	792	490	350	14 116	617	14 900
5	43			11	169	986	469	350	16 512	672	16 631
10	51			9	255	971	586	536	15 108	749	12 200
10	39			13	248	958	593	416	14 487	732	15 476
bronchial epithelial cells[b]	4 (7)	105 (195)	25 (22)	170 (300)				5 (19)			880 (1210)

[a] Eicosanoids not listed in the table were not detected. [b] The first number is for unstimulated cells, and the number in parentheses is for stimulated cells. Values are for 0.47 million cells per sample stimulated in 200 μL of Hank's balanced salt solution.

MS (19 pg on-column) (Table 2). Coefficients of variation ranged from 2.8 to 28.2%. We also analyzed three independent serum aliquots (10 μL), and the coefficient of variation ranged from 3.7 to 13.7%.

We then analyzed the set of endogenous eicosanoids present in mouse serum in order to test the AMPP derivatization method on a complex biological sample. Representative LC−ESI-MS/MS traces are shown in the Supporting Information Supplementary Material Figure 7 for a relatively high abundant serum eicosanoid (TxB_2) and a relatively low abundant eicosanoid ($PGF_{2\alpha}$). Traces for the full set of eicosanoids and internal standards are shown in the Supporting Information Supplementary Material Figure 3. Eicosanoids levels are shown in Table 3 for multiple runs and with different volumes of serum analyzed.

As another example of low-level eicosanoid analysis in a complex biological matrix, we analyzed eicosanoid formation in calcium ionophore stimulated primary bronchial epithelial cells. Results are summarized in Table 3, and selected ion traces for the full set of eicosanoids are shown as Supplementary Material Figure 4 in the Supporting Information.

Finally, we cross-validated the AMPP method with a commercially available antibody-based assay kit. We analyzed PGE_2 production by Rat 3Y1 cells and compared the results obtained via LC−ESI-MS/MS analysis of AMPP amides with those obtained from the EIA kit. Results are summarized in the Supporting Information Supplementary Material Figure 8. There is strong agreement between the two methods.

CONCLUSIONS

We developed a simple derivatization procedure to convert carboxylic acids to AMPP amides and showed that this significantly improves the sensitivity for detection of eicosanoids by LC−ESI-MS/MS. Antibody-based quantifications of eicosanoids have a limit of quantification around 1 pg, and the method requires that each analyte be quantified in a single assay well. Thus, if 12 eicosanoids are to be analyzed, one requires a sample containing ~12 pg of each species. The AMPP amide method disclosed in the current study can detect all 12 eicosanoid species in a single sample containing ~0.3−1 pg of each eicosanoid and is thus more than an order of magnitude more sensitive than antibody based detection. Previously reported LC−ESI-MS/MS detection of underivatized eicosanoids in negative ion mode provide a limit of quantification in the 10−20 pg range[1–3] (see also our data in Table 1), and thus our method is more than an order of magnitude more sensitive than these methods. We are currently studying the use of AMPP to improve the detection sensitivity for other oxygenated fatty acids including cysteinyl-leukotrienes, lipoxins, resolvins, and protectans. Analysis of the full spectrum of fatty acids should be feasible based on the results with AA reported in this study. This new method based on AMPP amides should find widespread use in the quantification of lipid mediator levels where sample supply is limited.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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