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Targeted quantitative analysis of eicosanoid lipids in biological samples using liquid chromatography-tandem mass spectrometry

Clementina Mesaros¹, Seon Hwa Lee², and Ian A. Blair^{1,*}

¹Centers for Cancer Pharmacology and Excellence in Environmental Toxicology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6160, USA

²Department of Bio-analytical Chemistry, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba-ku, Sendai 980-8578, Japan

Abstract

The eicosanoids are a large family of arachidonic acid oxidation products that contain twenty carbon atoms. Cyclooxygenase (COX)-derived eicosanoids have important roles as autacoids involved in the regulation of cardiovascular function and tumor progression. Lipoxygenase (LO)-derived eicosanoids have been implicated as important mediators of inflammation, asthma, cardiovascular disease and cancer. Cytochrome P-450 (P450)-derived eicosanoids are both vasodilators and vasoconstrictors. There is intense interest in the analysis of reactive oxygen species (ROS)-derived isoprostanes (isoPs) because of their utility as biomarkers of oxidative stress. Enzymatic pathways of eicosanoid formation are regioselective and enantioselective, whereas ROS-mediated eicosanoid formation proceeds with no stereoselectivity. Many of the eicosanoids are also present in only pM concentrations in biological fluids. This presents a formidable analytical challenge because methodology is required that can separate enantiomers and diastereomers with high sensitivity and specificity. However, the discovery of atmospheric pressure ionization (API)/MS methodology of electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and electron capture (EC) APCI has revolutionized our ability to analyze endogenous eicosanoids. LC separations of eicosanoids can now be readily coupled with API ionization, collision induced dissociation (CID) and tandem MS (MS/MS). This makes it possible to efficiently conduct targeted eicosanoid analyses using LC-multiple reaction monitoring (MRM)/MS. Several examples of targeted eicosanoid lipid analysis using conventional LC-ESI/MS have been discussed and some new data on the analysis of eicosanoids using chiral LC-ECAPCI/MS has been presented.

Keywords

Liquid chromatography-mass spectrometry; Lipids; Fatty acids; Electrospray ionization; Electron capture atmospheric pressure chemical ionization; Hydroxyeicosatetraenoic acids; Isoprostanes; Stable isotopes

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*Corresponding author: Center for Cancer Pharmacology, University of Pennsylvania School of Medicine, 854 BRB II/III, 421 Curie Boulevard, Philadelphia PA 19104-6160. Tel: 215-573-9880. Fax: 215-573-9889. ianblair@mail.med.upenn.edu.

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1. Introduction

Arachidonic acid is an important fatty acyl component of the lipidome (<http://www.lipidmaps.org/>), which is found esterified in sterol lipids and at the *sn*-2 position of glycerolipids and glycerophospholipids [1,2]. It is released by lipases [3] and converted to bioactive eicosanoids by COXs and LOs [4]. COX-2 can also convert arachidonic acid present in monoglycerides, to esterified eicosanoids [5], whereas 12-LOs, and 15-LOs can convert arachidonic acid present in glycerolipids, glycerophospholipids, sterol lipids, and complex lipid-protein assemblies to esterified HPETEs [6-8]. P450s can convert free and esterified arachidonic acid to epoxyeicosatrienoic acids (EETs) [2,9,10]. Finally, ROS can convert both free and esterified arachidonic acid through non-enzymatic processes to isoPs and HPETEs [11,12]. HPETEs formed enzymatically or non-enzymatically on esterified lipids are reduced to the corresponding hydroxyeicosatetraenoic acids (HETEs) by peroxiredoxin VI (PrxVI) [13] or phospholipid hydroperoxide glutathione peroxidase (GPx4) [14]. They are subsequently released by lipases as the corresponding free non-esterified HETEs [15]. HPETEs formed from free arachidonic acid are reduced to HETEs by glutathione transferases and peroxidases [14-16]. The resulting free HETEs from both enzymatic and non-enzymatic pathways can then be converted to oxo-eicosatetraenoic acids (oxo-ETEs), which form glutathione (GSH)-adducts [17]. Alternatively, esterified and free HPETEs undergo homolytic decomposition to highly reactive bifunctional electrophiles, which damage cellular DNA [18-21], RNA [22], proteins, and peptides [23,24]. Chiral lipidomics analysis of the free and esterified HETEs can provide insight into the structures of lipid precursors that cause this damage, helping to elucidate the roles that lipids play in oxidative stress [25].

The eicosanoids (from the Greek *eikosa*, which means twenty) are a large family of arachidonic acid oxidation products that contain twenty carbon atoms. Many of the eicosanoids are potent lipid mediators of biological processes [26]. COX-derived eicosanoids such as prostaglandins (PGs) and thromboxanes (TXs) have important roles as autacoids involved in the regulation of cardiovascular function [27] and tumor progression [28]. In contrast, LO-derived eicosanoids such as the leukotrienes (LTs) have been implicated as important mediators of inflammation, asthma, cardiovascular disease and cancer [29]. P450-derived EETs and their hydration products, the dihydroxyeicosatrienoic acids (DHETs), are vasodilators [30], whereas P450-derived 20-HETE is a vasoconstrictor [31]. Some ROS-derived isoPs have been implicated as mediators of vasoconstriction in different vascular beds, as smooth muscle cell mitogens, and as mediators of monocyte adhesion to endothelial cells [32,33]. However, the intense interest in isoPs stems primarily from their utility as biomarkers of oxidative stress [33,34]. Enzymatic pathways of eicosanoid formation are regioselective and enantioselective, whereas ROS mediated eicosanoid formation proceeds with no stereoselectivity (Scheme 1). Many of the eicosanoids are also present in only pM in biological fluids. This presents a formidable analytical challenge so that a methodology is required that can analyze enantiomers and diastereomers with high sensitivity and specificity. The analysis of low pM concentrations of eicosanoid enantiomers is particularly challenging [35].

Non-enzymatic HPETE formation results from abstraction of a *bis*-allylic hydrogen atom by ROS [36]. The resulting allylic radical adds molecular oxygen at the terminal allylic carbon atom. When the hydrogen from C-13 is abstracted from arachidonic acid, this results in the formation of 15-HPETE. Similarly, the other two *bis*-allylic hydrogens can be abstracted and two more regioisomer HPETEs are formed (Scheme 2). However, rearrangement of the allylic radical also results in changes to the double bond geometry so that two different stereoisomers (5*Z*,8*Z*,11*Z*,13*E* and 5*Z*,8*Z*,11*E*,13*E*) can be formed (Scheme 2). The addition of molecular oxygen occurs with no stereoselectivity so that two racemic isomers are formed. An allylic radical at C-13 can also add molecular oxygen to allylic carbon terminus at C-11 so that additional four isomers are produced. Allylic radical formation at C-10 and C-7 result in the

formation of eight more stereoisomers. Therefore, free radical mediated lipid hydroperoxide formation using arachidonic acid as substrate can lead to the formation of 24 different HPETE isomers (Scheme 2). In contrast COX- and LO-mediated HPETE formation is highly stereoselective. For example, COX-2 converts arachidonic acid to 11(R)-, 15(S)-, and 15(R)-HPETE [17] and 15-LO-1 converts it to 12(S)- and 15(S)-HPETE [4] (Scheme 2). Thus, chiral liquid chromatography (LC) methods are necessary in order to distinguish between enzymatic and non-enzymatic pathways of eicosanoid formation [37].

2. Eicosanoid assay methodology

Enzyme-linked immunosorbent assay (ELISA) methods have been widely used to quantify eicosanoids [38] but the questionable specificity when so many isomers can potentially cross-react with the various antibodies has limited their utility. High pressure liquid chromatography (HPLC) methods coupled with UV detection are useful for analyzing relatively high abundance eicosanoids that have suitable chromophores [7,39], such as HPETEs or HETEs.

In some cases, derivatization coupled with fluorescence detection has been used [40]. However, care has to be taken to ensure uniform derivatization occurs and that no interfering substances are introduced by the derivatization process. Alternatively, HPLC-UV analysis of high concentrations of unlabeled standards mixed with radiolabeled metabolites derived from ^{14}C -labeled arachidonic acid has also been employed [41]. Capillary electrophoresis has been used with some success [42] but it is not amenable to the analysis of all classes of the eicosanoids. Gas chromatography-mass spectrometry (GC-MS) in combination with electron capture negative chemical ionization (ECNCI) was commonly used in the past for eicosanoid analyses [43]. Extremely tedious derivatization procedures are required, making this technique very time-consuming for the analysis of large sample numbers. Furthermore, many of the eicosanoids are thermally unstable (HETEs, DHETs), so they cannot be readily analyzed by this technique. Nevertheless, the GC-MS methods were the first to allow larger number of the samples to be analyzed with high specificity and sensitivity [44-46]. Discovery of the API-MS techniques of ESI [47] and APCI [48] and ECAPCI [49] has revolutionized our ability to analyze endogenous eicosanoids [50,51]. Lipid chromatography (LC) using reversed phase solvents with microbore columns or normal phase solvents with chiral columns is now capable of separating complex mixtures of eicosanoid regioisomers and enantiomers. Advanced tandem MS technology can provide the highest sensitivity and specificity as well as the virtual separation of the isomers that LC is unable to resolve. The sample preparation for LC-MS analysis of eicosanoids usually consist of a single solid-phase extraction (SPE) or liquid-liquid extraction (LLE) step with or without one-step derivatization, which saves time and decreases losses of analytes.

LC-MS alone does not permit accurate quantitative analysis of eicosanoids in various biological samples. In contrast, stable isotope dilution LC/MS/MS represents one of the most specific and sensitive methods available for the analysis of eicosanoids. However, ion suppression can lead to inaccuracies if analyses are being conducted that approach the limit of detection when stable isotope analog internal standards are used. When no internal standard are used, the amount of suppression at different elution times can vary from sample to sample [52,53]. As noted above for deuterated standards, this can lead to the generation of erroneous results for individual eicosanoids.

Recent advances in targeted LC-MS/MS analysis of eicosanoids are detailed in the following sections.

3. Analysis of eicosanoids by targeted LC-MS

LC separations of eicosanoids can now be readily coupled with API ionization, CID and MS/MS making it possible to efficiently conduct targeted eicosanoid analyses [51]. Product ion profiles are often diagnostic for particular regioisomers. In addition, eicosanoid regioisomers such as PGD₂ and PGE₂ can be separated by LC, which makes it possible to readily analyze large number of samples [54]. There are many publications describing the LC-MC analysis of eicosanoids. However, relatively few publications reported the detection of more than one class of eicosanoid [54-59].

The highest sensitivity can be attained for the analysis of eicosanoids involves the use of LC-MRM/MS. Maximal specificity is obtained when the MRM transition is between an intense parent ion which contains the intact molecule (M) and structurally significant product ion. An example of this desirable situation arises with HETEs, where product ions are formed through α -cleavage adjacent to a double bond [50]. In some cases, this is not possible because isomeric eicosanoids sometimes produce similar product ion profiles. An example of this less desirable situation arises with isoPs. This means that rigorous LC separation of the various isomers is required [60]. Most LC-MRM/MS methods that have been reported employ ESI in the negative ion mode, where the parent ion arises from deprotonation of the eicosanoid molecule to give an ion corresponding to $[M-H]^-$. LC has generally been performed using reversed-phase stationary phases coupled with aqueous mobile phases [54,61,62]. Quantification is conducted using stable isotope dilution methodology with ²H-labeled eicosanoid analogs as internal standards (ISs). Deuterium-labeled analogs are not ideal as ISs because they are often separated from their protium forms during complex LC separations. This means that there could be differential suppression of ionization of the deuterated IS when compared with the eicosanoid analyte in biological samples leading a potential for inaccuracy in quantitation. However, ¹³C-eicosanoid analogs are not commercially available so the ²H-labeled analogs represent the current state-of-the-art as ISs and they are certainly preferable to the use of structural analogs. Quantification is performed by constructing calibration curves for each analyte. Standard solutions of different concentrations are made by serial dilution from commercially available standard eicosanoids and they are spiked with the same amount of the ²H labeled IS as the samples to be determined. These different solutions are analyzed by the LC-MS system under exactly same conditions as the unknown samples. The ratio of the area of the authentic standard over the area of the IS is plotted vs the concentration of the added standard. Rigorous quantification of eicosanoids by stable isotope dilution LC-MRM/MS assays is difficult because eicosanoids are normally present at low pM concentrations in the biological sample of interest. One way to overcome this problem is to construct standard curves in water and in the biological fluid of interest. The two curves should be parallel and the intercept on the y-axis of the standard curve for the biological sample should correspond to the endogenous concentration of the eicosanoid of interest.

An illustrative reversed-phase LC-MS method for the analysis of a large number of eicosanoids has been reported by Deems et al. [54]. The method has several advantages, including very short chromatographic runs (16 min); a large number (60 eicosanoids total) of products detected in a single run and no need of the extra steps for derivatization commonly employed in GC-MS. SPE columns were employed to separate the eicosanoids from cell incubation media or cell lysates. It is noteworthy that the cells were harvested in methanol in order to prevent artifactual eicosanoid biosynthesis. A small number of stable isotope ISs was added in the beginning of the sample work-up to compensate for potential losses of individual analytes. It was possible to separate seven PGs in the first 13 min of the chromatographic run (Fig. 1A). The peaks were sharp with half-widths of less than 10 sec. In the same segment of the chromatogram, the HETEs regioisomers eluted (Fig. 1B). However, they had no overlapping ions with the PGs and so they could be readily analyzed. The complexity of the chromatogram

makes it difficult to conduct accurate quantitation of each HETE. This method is suitable for the analysis of eicosanoid biosynthesis when relatively large amounts are present. However, further chiral analysis is required to distinguish individual enantiomers.

A stable isotope dilution reversed phase LC-MS method was reported for the analysis of a wider range of eicosanoids, which included EETs and DHETs [61]. This method is capable of analyzing 19 eicosanoids using 7 deuterated ISs. The HETE regioisomers were more efficiently separated and each of the EETs had ^2H labeled analog ISs, although again it was not possible to conduct a chiral analysis of the EETs using this method. Zhan et al. [62] developed a similar procedure for eicosanoid extraction and analysis from human intrauterine tissues. The 31 min LC run time coupled with MRM transitions allowed the detection of 12 eicosanoids. Only two ^2H -labeled analogs ISs were employed, which could be problematic for accurate quantitation of the more problematic eicosanoids. However, great care was taken to develop an efficient extraction of the eicosanoids from the tissue samples. It was found that ethanol disrupted protein binding and facilitated more reproducible eicosanoid recovery.

The work of Kiss et al. [63] presents the detection of 44 eicosanoids from lung tissue by capillary liquid chromatography with dual online photodiode-array and tandem mass-spectrometric detection. The eicosanoids detected in a single 50 min run by this method belonged to all classes produced enzymatically. They used 19 ^2H -labeled analogs ISs and 2 non-deuterated standards. The extraction procedure was developed for rabbit lung homogenate and used a two-steps elution to eliminate the interfering substances on the SPE cleaning-up. The identification of some eicosanoids with identical MS^2 spectra was improved by UV spectra. Great care was taken to check for signal suppression by diluting standards with eluate from the final step of SPE of the biological matrix and comparing with same standards diluted with water or with the mobile phase. The complexity of the chromatogram makes it difficult to conduct accurate quantitation for all of the eicosanoids detected in the same run. This method is suitable for the analysis of eicosanoid biosynthesis when relatively large amounts are present. Here too, further chiral analysis is required to distinguish individual enantiomers.

4. Analysis of eicosanoids by targeted chiral LC-ECAPCI/MS

The pioneering work of Horning et al. [48] showed that ionization under APCI conditions was initiated by the $\text{N}_2^{+\bullet}$ radical cation, which in turn was formed by collision of high-energy electrons from the corona discharge with the nitrogen sheath gas. We found that the low-energy electrons generated in the APCI source through this process were found to ionize eicosanoid-PFB derivatives analytes through dissociative electron capture [49]. The ionization process is analogous to that occurring in a conventional chemical ionization source during GC-ECNCl/MS analysis (Fig. 2). A 25- to 100-fold increase in sensitivity for eicosanoids was obtained by ECAPCI/MS when compared with normal APCI methods [49]. Furthermore, LC-ECAPCI/MS analyses of eicosanoid PFB-derivatives can be conducted using normal phase solvents and chiral columns without loss of sensitivity. This makes it possible to separate complex mixtures of eicosanoid enantiomers and to quantify them with very high sensitivity using stable isotope dilution methodology [35,37,64]. Derivatization is reminiscent to that used for GC-ECNCl/MS analyses of eicosanoid PFB-derivative [45] but there is no need for further derivatization of the hydroxyl groups or ketones. Furthermore, it is not necessary to purify the PFB-ester derivatives prior to LC-MS analysis. Therefore, the work-up procedure is much faster than that required for GC-ECNCl/MS. LC-ECAPCI/MS is particularly efficient when used with fast liquid-liquid extraction procedures rather than more tedious SPE, which is commonly used for conventional LC-MS analyses of eicosanoids. A typical chromatogram obtained from a targeted chiral LC-MRM/MS analysis of 17 eicosanoids and 9 ^2H labeled analog ISs using ECAPCI is shown in Fig. 3. Detection limits for individual eicosanoids were found to be in the fg range. The LC-ECAPCI/MS method made it possible to unequivocally show that 11

(*R*)-, 15(*S*)-, and 15(*R*)-HETE were produced by COX-2 in similar amounts to PGE₂ by epithelial cells in culture (Fig. 4) [17]. The method was also employed to show that 15(*S*)-HETE was converted to 15-oxo-EETE by the epithelial cells (Fig. 4).

Chiral LC-ECAPCI/MS can also be readily employed for the quantification of eicosanoids in tissue samples from different organs. This can provide information on the site of biosynthesis of PG and HETE isomers. By first extracting esterified lipids with a simple Bligh and Dyer [65] method, and then dividing the organic extract in two and conducting an alkaline hydrolysis, the total and free eicosanoid profile can be obtained. This approach was employed to examine eicosanoid production in the dog heart (Fig. 5). A targeted chiral analysis unequivocally demonstrated the production of the 12(*S*)-HETE consistent with a previous report which had shown that the 12-LO pathway was activated and up-regulated during myocardial ischemia/reperfusion injury [66]. An achiral LC-MS methods would have detect the 10-fold increased in the levels of the 12-HETEs enantiomers compared with the others HETEs regioisomers, but would not have implicated the 12-LO pathway.

There are very few published reports describing the analysis of HETEs in human urine [67-70]. The ability to conduct profiling of urinary HETEs could provide an important screening tool for different diseases and metabolic processes. For example, Sacerdoti et al. [67] used LC purification followed by GC-MS to quantify the P450-derived ω -oxidation P450 products of arachidonic acid (16-, 17-, 18- and 19-, and 20-HETE) in the urine of cirrhotic subjects. The findings that the 20-HETE was excreted primarily as its glucuronide conjugate was in accordance with our previous finding [46]. It was shown that the other urinary HETE ω -oxidation products were also glucuronidated. Furthermore, levels of 20-HETE (but not the other HETEs) were elevated in the cirrhotic patients [67]. Subsequently, it was shown that both 15- and 20-HETE are converted to glucuronide conjugates by hepatic UDP-glucuronyl transferases [71]. It is noteworthy that neither 20-HETE nor its glucuronides metabolite were detected in rat urine [68].

The enantioselective analysis of other urinary HETEs has been a significant analytical challenge for LC-MS methodology because of the presence of many interfering compounds that are preset in urine. In fact, there is only one report describing the use of LC-MRM/MS to quantify 12(*S*)-HETE in the urine of diabetic subjects [72]. However, the method is impractical for large numbers of samples as it requires chromatographic runs times of > 60 min/sample. In addition, the method provides only partial separation of the 12(*S*)- and 12(*R*)-HETE enantiomers with peak widths of > 5-min. We recently discovered that by adjusting the pH of the urine samples to 6.0, a simple liquid-liquid extraction is highly selective for extracting 5-, 12-, and 20-HETEs that removes interfering substances from the urine. Using this extraction method coupled with the chiral LC-ECAPCI/MRM/MS methodology [64], it was possible to analyze 5(*R*)-, 5(*S*)-, 12(*R*)-, 12(*S*)- and 20-HETE in a single analysis (Fig. 6). Chromatographic peaks were typically < 1 min wide. As noted above, we previously used an extensive purification procedure coupled with GC-MS to show that the urinary 20-HETE (an achiral metabolite) is primarily excreted by humans as its glucuronide conjugate [46]. We have now re-confirmed our original finding using this much simpler LC-ECAPCI/MS/MS assay. In contrast, the enantiomers of 5- and 12-HETE were excreted primarily in their un-conjugated forms. Interestingly, 12(*S*)- and 20-HETE (as its glucuronide) were the major HETEs found in the urine of smokers.

5. Targeted LC-MS analysis of isoPs

F₂ isoPs are eicosanoids that are isomeric with the PGF_{2 α} (one of the COX products). They arise from ROS-mediated oxidation of arachidonic acid through the intermediate formation of an allylic radical (Fig. 7). This results in the formation of four classes of isoprostanes as shown

in Fig. 7. Each F₂ isoP class has a cyclopentane ring (like PGF_{2α}) with a *cis* configuration of the side chains on the cyclopentane ring which distinguishes them from the *trans* configuration of the COX-derived PGF_{2α}. Having three chiral centers, each of the classes can comprise eight racemic diastereomers, adding the total number of possible isomers to 64. There are currently two nomenclatures used for isoprostanes. One system, which was developed by Taber et al. [73] considers the position of the initially formed peroxy radical for the determination of each class. A second system, which was developed by Rokach et al. [74] is based on the omega-carbon as starting reference. We have used here the Rokach nomenclature system. IsoPs were validated as reliable biomarkers of oxidative stress in a multi-laboratory collaboration [75, 76]. The studies focused on 8-*iso*-PGF_{2α} (class III) and 8,12-*iso*-PGF_{2α} VI (class VI) 8-*iso*-PGF_{2α} also known as 8-*epi*-PGF_{2α} and iPF_{2α}-III is the most studied of the isoP isomers. Significant elevations of both isomers were shown to occur in the urine of rats treated with carbon tetrachloride.

It is remarkable that since the first reports of esterified isoPs [77,78] the interest in the detection of the isoPs in urine had generated almost 400 papers. LC-MS methods used for isoP analysis employ primarily reversed phase LC coupled with positive ESI/MS. 8-*iso*-PGF_{2α} is the most frequent isoP to be analyzed and there are relatively few reports that are detecting more than one isoP in a single LC-MS analysis. Each class of isoP has forms a specific product ion during LC-MS/MS analysis [60] making it possible to differentiate the different classes. The characteristic product ion for class III is *m/z* 193 and for class VI is *m/z* 115. However there are potentially 16 class III and 16 class VI isoP isomers so it is important to conduct efficient LC separations. Furthermore, class VI isomers also have a product ion at *m/z* 193 that is approximately ten times less intense as the one from class III.

The first publication [79] reporting the use of LC-MS for quantification of isoPs in urine used reverse phase LC coupled with ESI/MS. The method used only 1 mL urine and the clean-up procedure using SPE columns gave quantitative recovery of the isoP. The chromatographic runs are fairly short and combined with the short SPE purification makes this procedure very useful.

A comprehensive review by Schwedhelm et al. [80] describes samples preparation techniques and compare GC-MS methods with the most recent LC-MS/MS methods. They focus mostly on the 8-*iso*-PGF_{2α}, pointing out the difficulty to detect only one isoPs isomer, without IAC preparation. The big differences in concentration for different isoP classes in urine is pointed out, together with the baffling fact that in class III of isoP was found to have the higher concentration in human urine, but in a clinical setting other classes were found to be more abundant. This work offers a great starting point for a scientist trying to get a general idea about the isoPs' history but also about the part of the isoPs in human disorders.

Among the recent publications detailing isoP quantification in urine, Yan et al. [81] have reported the detection of three class III isomers and two class VI isomers. Concentrations determined by LC-MS were then correlated with those obtained by ELISA measurements. Interestingly, the amount determined by LC-MS as approximately half that found by ELISA. The report by Yan et al. is not the only example of this problem, which probably results from the cross reactivity of the antibody with other isoPs in the urines. LC-MS/MS methods are potentially more accurate than ELISA-based methodology because they can separate the individual isomers and reduce interference from the same class of isoP.

LC-MS/MS methodology, when accompanied by efficient LC separation has the selectivity to detect most of the 64 possible isoP isomers. For examples (as shown in Fig 8, for a urine sample) there are two other very abundant peaks (retention time 11.5 and 15.8 min) that are most likely isoprostanes from class VI since they exhibit the specific fragment at *m/z* 115. Unfortunately,

they could not be completely characterized due to the lack of authentic synthetic standards for the class VI isoPs.

Several groups have used immunoaffinity chromatography [82,83] for the purification of eicosanoids prior to analysis. Immunoaffinity purification can be regarded as a providing a “gold standard” for the purification of endogenous compounds when conducted under rigorous conditions [84]. Immunoaffinity columns for 8-iso-PGF_{2α} are commercially available. When these columns were coupled with stable isotope dilution LC-MS/MS methodology, excellent sensitivity and specificity could be obtained. Furthermore, relatively small urine volumes (0.3 mL) were required [82]. The specificity of this methodology is particularly useful for analyses by GC-MS when LC-MS/MS instruments are not available. However, care has to be taken to ensure there is no carry over from sample to sample. Columns should be checked for residual endogenous eicosanoids between analytical runs by analyzing the internal standard alone. Unfortunately, the method is somewhat limited for the isoPs because there are very few antibodies available for other structural analogs.

We have recently applied stable isotope dilution chiral LC-ECAPCI/MRM/MS methodology to the analysis of multiple isoPs in human urine (Fig. 8) after liquid-liquid extraction and PFB derivatization.. This has made it possible to show a significant increase in 8-iso-PGF_{2α} (class III) and 8,12-iso-PGF_{2α}-VI (class VI) in the urine of smokers compared with non-smokers. 8-iso-PGF_{2α} had a concentration of 0.6 ng/mg creatinine in a typical smoker's urine sample (Fig. 8) and 0.1 ng/mg of creatinine in a typical non-smoker's sample (data not shown). 8,12-iso-PGF_{2α}-VI had a concentration 1.9 ng/mg creatinine in a typical smoker's urine sample (Fig. 8) and 0.2 ng/ mg creatinine in a typical non-smoker's sample (data not shown).

6. Summary and conclusions

LC-MS has revolutionized our approach to the targeted analysis and quantitation of eicosanoids. This has made it possible to begin unraveling the role of eicosanoids in various disease processes. Using LC-MRM/MS procedures and stable isotope dilution it is now possible to quantify up to 60 eicosanoids in a single analytical run. ²H -labeled eicosanoid analogs are normally used as ISs. These are not ideal because they are often separated from their protium forms during complex LC separation. This means that there could be differential suppression of ionization of the IS when compared with the eicosanoid analyte leading a potential for inaccuracy in quantitation. However, ¹³C-eicosanoid analogs are not commercially available so the deuterium-labeled analogs ISs represent the current state-of-the-art. The availability of high sensitivity ECAPCI/MS has made it possible to conduct the analysis of endogenous eicosanoids using on-line chiral separations. It is anticipated that in the future it will be able to extend this methodology to the analysis of P450 metabolites - EETs and DHETs. The separation of individual deuterium labeled enantiomers and diastereomers will make it very difficult to conduct precise and accurate quantification of EETs and HETEs because the chiral separations lead to complex chromatographic profiles. Therefore appropriate ¹³C-isotope analogs will be required for use as internal standards.

One of the limitations of the LC-MRM/MS approach is the limited number of transitions that can be monitored. However, new instrumentation is being introduced that can address this issue. Improved peak shapes and more reproducible chromatographic retention times can be attained using high-pressure ultra performance LC (UPLC) [85]. In addition, instruments such as the Waters Xevo tandem mass spectrometer can conduct hundreds of MRM analyses when used in combination with UPLC. Unfortunately, chiral UPLC columns are not currently available, so these advances will be restricted to achiral analyses. Nevertheless, the ability to quantify hundreds of eicosanoids in a single analysis will provide an excellent screening tool so that more targeted chiral determinations can be conducted subsequently using LC-ECAPCI/

MRM/MS [64]. Such new methodology will provide an even more efficient way to further define the roles of eicosanoids in a large diversity of pathophysiological processes.

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Abbreviation List

AA, arachidonic acid
CID, collision induced dissociation
COX, cyclooxygenase
P450, cytochrome P-450
DHETs, dihydroxyeicosatrienoic acids
ECNCl, electron capture negative chemical ionization
ESI, electrospray ionization
ELISA, enzyme-linked immunosorbent assay
GC-MS, gas chromatography-mass spectrometry
HPLC, high pressure liquid chromatography
HETEs, hydroxyeicosatetraenoic acids
isoPs, isoprostanes
LTs, leukotrienes
LO, lipoxygenase
LC, liquid chromatography
LLE, liquid-liquid extraction
MRM, multiple reaction monitoring
oxo-ETEs, oxo-eicosatetraenoic acids
PFB, pentafluorobenzyl
PGs, prostaglandins
ROS, reactive oxygen species
SPE, solid-phase extraction

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Scheme 1.
COX-, LO-, P450-, ROS-, and EH-mediated formation of eicosanoids.

Scheme 2.

ROS-, COX-2-, and 15-LO-1-mediated HPETE formation

Fig. 1. High performance liquid chromatography (HPLC) eicosanoid chromatography on reverse-phase C18. The chromatography profiles of selected eicosanoid standards run on reverse-phase C18 HPLC. In each panel, the individual chromatograms produced by a given MRM pair have been overlaid. Each label lists the eicosanoid, retention time, and MRM transition that produced a given chromatogram. (A) Representative sample of prostaglandins and arachidonic acid (AA). (B) Representative sample of the hydroxyeicosanoids. Reprinted with permission from ref [60].

Fig. 2.
ECAPCI-MS analysis of 15(S)-HETE-PFB.

Fig. 3.

Targeted lipidomics using LC-ECAPCI/MS/MS for analysis of eicosanoids derivatives and 9 ISDs. MRM/MS chromatograms are shown for 17 eicosanoids and 9 deuterated ISDs.

Reprinted with permission from ref [64].

Fig. 4.

Targeted chiral lipidomics analysis of COX-2-derived eicosanoids from intact RIES cells treated with arachidonic acid (10 μM final concentration) after 10 min. Reprinted with permission from ref [17].

Fig. 5.
Targeted chiral lipidomics analysis of eicosanoids from dog heart tissue spiked with 1 ng of IS mixture of synthetic standards.

Fig. 6.

Typical LC-ECAPCI/MRM/MS chromatogram of HETEs in a 3 mL smoker's urine sample spiked with 1 ng of IS mixture of synthetic standards. The arrows indicate the corresponding enantiomers.

Fig. 7.

F₂ IsoP formation from arachidonic acid. Reprinted with permission from ref [33].

Fig. 8.

Typical LC-ECAPCI/MRM/MS chromatogram of isoPs in a 3 mL smoker's urine sample spiked with 1 ng of IS mixture of synthetic standards. The arrow indicates the chromatographic peak at 12.8 min, corresponding to the 8,12-iso-iPF_{2α}, which is less visible otherwise, because of the two very abundant peaks at 11.5 and 15.8 min.