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Determination of epoxyeicosatrienoic acids in human red blood cells and plasma by GC/MS in the NICI mode

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ABSTRACT

Epoxyeicosatrienoic acids (EETs) are cytochrome P450 metabolites of arachidonic acid involved in the regulation of vascular tone. Despite the importance of EETs in a variety of physiological effects, few methods have been developed to quantify them in human blood. This led us to develop a method by GC/MS with negative ion chemical ionization. As EETs are primarily located in phospholipids, red blood cells (RBCs) and plasma phospholipids were hydrolyzed with phospholipase A_2 after a solid phase extraction. Then, EETs were derivatized as pentafluorobenzyl esters, and [$^2H_8$]-arachidonic acid was used as internal standard for quantification. EETs were found to be at concentrations of 106 ± 37 ng mL^{-1} in plasma and 33.4 ± 8.5 ng/10^9 RBCs (mean ± S.D.) in 10 healthy volunteers. Their amount in RBCs was 3-fold that in plasma; both parameters proved to be well correlated.

1. Introduction

Epoxyeicosatrienoic acids (EETs) are endogenous bioactive lipid mediators synthesized from arachidonic acid (AA) by cytochrome P450 epoxygenase [1–3]. Monooxygenation of AA by cytochromes P450 leads to the formation of four cis-regioisomers: 5,6-, 8,9-, 11,12- and 14,15-EET, with 14,15-EET being the most abundant regioisomer [4]. The primary function of EETs is to act as autocrine and paracrine mediators in the cardiovascular and renal systems. Their metabolism, biochemical activities and actions on cellular functions have been the subject of recent reviews [5,6]. By modulating ion transport and gene expression, EETs trigger vasorelaxation and produce antiinflammatory and pro-fibrinolytic effects. They also play a role in the regulation of angiogenesis and tumor growth [7].

EETs have been identified in different tissues such as the liver [8], the kidney [9], the brain [10] or blood [4]. They are usually assumed to be produced in the tissues where they act, but their presence in blood suggests a supply to, at least, certain tissues. Recent studies in rats showed that EETs are present not only in plasma [4] and platelets [11] but also in red blood cells (RBCs) [12,13]. More than 90% of the EETs contained in plasma, RBCs or platelets are located in the *sn*-2 position of phospholipids [5]. The processes at the origin of phospholipids-bound EETs could be enzymatic by CYP epoxygenases or nonenzymatic by oxydative stress [14]. RBCs could represent potent reservoirs of EETs that may be released through activation of phospholipase A_2 (PLA_2) [14] and implicated in the control of microcirculation and rheological characteristics of the circulating blood [15].

Recent investigations have pointed to an emerging role for EETs in vascular function [16–21]. EETs are metabolized primarily by conversion to dihydroxyeicosatrienoic acids (DHET), a reaction catalyzed by soluble epoxide hydrolase (sEH) [6] and over the last years, considerable attention has been given to pharmacological inhibition of this enzyme [17,22,23] or sEH gene deletion [20] as a new therapeutic approach against hypertension [17,24,25], or cerebral ischemia [20]. Furthermore, genetic polymorphisms of CYPs involved in EET synthesis have been linked to hypertension in humans [26–28]. Therefore it would be of interest to be able to relate the blood EET levels to hypertension or cardiovascular effects. In a very recent study, low plasma levels of EETs were found to be associated with high levels of 20-HETE in renovascular disease [29]. However, reports on blood EET levels in patients are very scarce although their dosage is needed, not only to assess their real role in hypertension or cardiovascular dis-

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eases, but also to investigate the potential role of EET reservoir of RBCs.

Determination of eicosanoids requires very specific and sensitive methods because of their low levels in various tissues (for review, see [13,30,31]. Currently, GC/MS [32], LC/MS [10,33]) and LC/MS/MS methods [13,14,32] have been used to measure some of these eicosanoids in different tissues. Two methods have been described for determination of EETs bound to phospholipids in RBCs [13,14]. Both combined two successive chromatographic separations. Such procedures were required because substances from the biological matrix with the same m/z ratio and eluting at the same retention time as target eicosanoids interfered in LC/MS as in GC/MS. To solve this problem, purification of samples was achieved: Nakamura et al. [14] separated phospholipids from the lipid extract by normal-phase LC before the hydrolysis step and then separated and quantified EETs by LC/MS/MS. Jiang et al. [13] separated EETs released from phospholipids hydrolysis by LC/MS/MS and then quantified them by GC/MS after derivatization as PFB-esters and detection based on negative ion chemical ionisation (NICI). In fact, a high sensitivity and specificity can be obtained by GC/MS/NICI quantification [4,11,15]. The derivatization as PFB esters of the carboxylic group increases the specificity of detection by MS, compared to LC where no derivatization reaction occurs. Moreover, the GC peak of total EETs is only a few seconds wide while the separation of individual EETs achieved by LC spans over several min, decreasing its sensitivity when compared to GC/MS [13,34]. It has to be underlined that GC cannot separate the different EET regioisomers [32]. However, these methods, described for the determination of EETs in RBCs, are aimed to quantify each EET regioisomer in each class of phospholipids [13,14] and thus, are rather time-consuming and not well adapted to series dosages of total EETs only useful for clinical studies. An alternative to these methods can be a solid-phase extraction (SPE) of phospholipids or eicosanoids, before the LC/MS, LC/MS/MS or GC/MS analysis [10,32,33]. These considerations have led us to develop a sensitive and reliable GC/MS/NICI method including a preliminary purification step of phospholipids by SPE with a special emphasis on the blood sample preparation. Using this method, total RBC and plasma EETs have been quantified in 10 healthy volunteers as an initial validation.

2. Experimental

2.1. Chemicals

5Z,8Z,11Z,14Z,-eicosatetraenoic-5,6,8,9,11,12,14,15-d8 acid ([$^2H_8$]-AA: arachidonic acid-d8), (±)5(6)-epoxy-8Z,11Z,14Z-eicosatrienoic acid (5,6-EET), (±)8(9)-epoxy-5Z,11Z,14Z-eicosatrienoic acid (8,9-EET), (±)11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid (11,12-EET), (±)14(15)-epoxy-5Z,8Z,11Z-eicosatrienoic acid (14,15-EET) and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid (DHA) were purchased from Cayman Chemical (Spi-Bio, Montigny Le Bretonneux, F). (±)14(15)-Epoxy-5Z,8Z,11Z-eicosatrienoic-5,6,8,9,11,12,14,15-d8 acid (14,15-[$^2H_8$]-EET) was from Biomol (Exeter, UK). [1-^{14}C]-5,8,11,14-eicosatetraenoic acid (^{14}C-AA) at the specific activity of 2.07 GBq/mmol, was purchased from Amersham Biosciences (UK).

Sylon BFT (bis(trimethylsilyl)trifluoroacetamide:trimethylchlorosilane, 99:1, v:v) was from Supelco (Bellefonte, PA, USA); *m*-chloroperbenzoic acid (*m*-CPBA), triphenylphosphine (TPP, 99%), PLA_2 (*Naja Mossambica Mossambica*), *N*,*N*-diisopropylethylamine and α-bromo-2,3,4,5,6-pentafluorotoluene were from Sigma–Aldrich (L'Isle d'Abeau, F). Acetonitrile, methanol, propanol, diethyl ether, hexane and chloroform (all pure analytical grades) were purchased from Carlo Erba (Carlo-Erba réactifs, Val de Reuil, F). Ethyl acetate (for analysis) was from Merck (Fontenay sous Bois, F).

2.2. ^{14}C-EETs synthesis and purification

Ten nmoles of a ^{14}C-AA solution were incubated for 15 min and at room temperature with one equivalent of *m*-CPBA in 100 μL of dichloromethane. Then, dichloromethane was evaporated under a stream of oxygen-free nitrogen, the residue was dissolved in ethanol, stored at −20 °C until use and separated by RP-HPLC as previously described [34]. ^{14}C-EETs were recovered in 100 μL MeOH and stored at −80 °C for further use in Solid Phase Extraction (SPE) development.

2.3. Blood samples preparation

Blood was obtained from 10 healthy volunteers after informed written consent. The study has been approved by the local ethic committee of the University Hospital of Brest.

Blood (5 mL) was collected on BD Vacutainer® tubes containing heparin and layed immediately on ice. RBCs were separated from plasma by centrifugation at 800 g at 4 °C for 10 min. The white blood cells were removed by aspiration and RBCs were washed three times by a physiological salt solution containing 0.1 mM TPP. TPP was used to quench free radicals and to prevent radical-propagated transformations of polyunsaturated structures [35,36]. Erythrocytes (0.5 mL) were resuspended in a known volume of physiological salt solution prior to the sampling of an aliquot for counting. After centrifugation of aliquots at 800 g and 4 °C for 10 min, the supernatant was removed. Then the samples were stored at −80 °C for further phospholipid extraction.

2.4. Phospholipids extraction, purification and hydrolysis

2.4.1. Extraction

The prepared RBCs were lysed by addition of an equivalent volume of water and centrifuged at 4000 × *g* at 4 °C for 5 min. The phospholipids from RBCs and plasma were separately extracted by mixing with 4 mL of $CHCl_3/CH_3OH$ (2:1) [37] containing 0.1 mM TPP on a rotary shaker for 1 H. The samples were centrifuged at 4000 × *g* at 4 °C for 15 min.

2.4.2. Purification

Prior to the separation by SPE of the phospholipids from the other lipids, different columns were tested to determine their efficacy: Sep Pak C18 and Oasis® MCX (Waters, Milford, MA), Bond Elut Jr-C18-Aminopropyl, -C18, -C18+ and −Sax, at 500 mg in size) (Varian, Harbor City, CA); the C18-aminopropyl was selected because it was the most efficient for separation of phospholipids from other lipids as for recovery of EETs. The phospholipids were extracted according to the procedure by Vaghela et al. [38]. The chloroform layer was transferred to the SPE column, pre-conditioned with 3 mL ethanol and 5 mL water. The first compounds to be eluted with 4 mL of a mixture of chloroform/propanol (2:1 v:v) were the neutral lipids. Then, the fatty acids were eluted with 4 mL of 2% acetic acid in diethyl ether. Finally, the phospholipids were eluted with 4 mL methanol. EET content was determined at each step of elution after hydrolysis with phospholipase A2 (see below). The elimination of free fatty acids was monitored by using ^{14}C-AA and ^{14}C-EETs that were separately applied to the SPE column. The recovery of radioactivity in every elution phase was followed with a TriCarb Liquid Scintillation Counter (PerkinElmer Life, MA, USA).

2.4.3. Hydrolysis

The internal standard (250 ng [$^2H_8$]-AA) was added to each methanolic sample. The methanolic fraction was dried under a gentle stream of oxygen-free nitrogen. The phospholipids were hydrolyzed by 10 units of PLA_2 in 1 mL Tris buffer at pH 8.9, for 12 min at 37 °C. The reaction was stopped by addition of 100 μL of acetic acid. The supernatants were combined and dried under a gentle stream of oxygen-free nitrogen.

2.5. GC/MS analysis

2.5.1. Derivatization

The residue of extraction was resuspended in 100 μL acetonitrile. To form PFB-esters [32], the carboxylic groups were reacted with 10 μL of α-bromo-2,3,4,5,6-pentafluorotoluene in the presence of 20 μL of *N*,*N*-diisopropylethylamine for 15 min at room temperature. The solvent was dried under a gentle stream of nitrogen and the residue was dissolved in 2 mL of water. Extraction was performed with 2 × 2 mL of ethyl acetate. The combined organic aliquots were dried under nitrogen gas and redissolved in 100 μL acetonitrile. To form TMS-ethers on the potential hydroxyl groups of oxygenated derivatives of fatty acids, 100 μL of Sylon BFT were added and let to react for 1 h at 60 °C. Eventually, the solution was dried under nitrogen gas prior to re-dissolution of the sample in 100 μL of hexane.

2.5.2. GC/MS analysis

The apparatus used for analysis by GC/MS was a Hewlett-Packard 5873 Mass Selective Detector interfaced to a Hewlett-Packard 6890 Series+ gas chromatograph (Agilent, Les Ulis, France) and equipped with an HP-5MS capillary column (0.25 mm i.d., 30-m-long and 0.25-μm-thick film; J & W Scientific). Other columns from different polarities have been tested: CP sil-5 CB (Varian-Chrompack, Middelburg, NL), HP5 and HP50 (Agilent, Les Ullis, F), and RSL 500 (Bio-Rad RSL, Eke, B). The injected volume of sample was 2 μL in the splitless mode on a single taper deactivated liner (Agilent, Les Ulis, France). The carrier gas was Helium IC at 1 mL min^{-1}. The temperatures of injection port and interface were 250 and 280 °C, respectively. The oven temperature was set at 60 °C for 5 min, risen to 230 °C at the rate of 10 °C min^{-1}, then to 290 °C at the rate of 1 °C min^{-1} and held at 290 °C for 3 min. However, different temperature gradients have also been tested, rising temperature from 230 to 290 °C at the various rates of 10, 8, 6, 4 or 2 °C min^{-1}. The mass spectrometer ion source and the analyzer were set at 250 and 100 °C, respectively. The compounds were ionized by NICI with methane as reagent gas at 40 mL min^{-1} in flow rate. Ionization energy and trap current were 123 eV and 49 μA, respectively. The data were collected in both total ion (TIC, *m/z* 100–500) and selected-ion monitoring (SIM) chromatograms. The ions at *m/z* 319, 311 and 327 were used for EETs, [$^2H_8$]-AA and 14,15-[$^2H_8$]-EET respectively.

2.6. Calibration and validation of the method

A five-point calibration plot (50, 100, 150, 200, and 250 ng mL^{-1}, corresponding to 1–5 ng injected) was drawn from a mixture of the four EETs added to either plasma or RBC hemolysate after SPE. The ratios of peak areas of the analytes/internal standard were calculated and blanks (corresponding to endogenous EETs) were substracted. The calibration curves were drawn by linear regression analysis. The linearity was tested for concentrations in the range 0–50 μg mL^{-1} which corresponded to 0–100 ng of injected EETs. Analyte recovery was determined by addition of a mixture of all eicosanoids as standards at 3 concentrations in RBC hemolysate after SPE (20, 100 and 200 ng mL^{-1}) and comparison of the found concentration against the added one. Samples (n = 10) at low (plasma) and high (RBCs) levels of eicosanoids were used to determine the intraday and interday precision. Samples for the intra-day precision were prepared in parallel on the same day and stored at 4 °C before injection while samples for the inter-day precision were prepared extemporaneously on each day from RBC hemolysate stored at −80 °C. The limit of qualitative detection (LOD) and the limit of quantitative detection (LOQ) were tested with standard solutions (without the biological matrix). In accordance with the definition by Currie [39], LOD was set as 3 times the signal/noise ratio and LOQ was the lowest concentration of analyte that produced a coefficient of variation of 20% or less. The lower limit of quantification (LLOQ) was determined in a biological sample (hemolysate) as the lowest concentration of added EET which was recovered with a percent of deviation lower than 10%.

3. Results and discussion

In this study, we describe a GC/MS/NICI method including a preliminary step of purification of phospholipids by SPE, to determine total EETs in RBCs and plasma phospholipids from human blood.

3.1. GC/MS/NICI analysis

Fig. 1A presents the chromatogram of a mixture of the four standard regioisomers EETs (5,6-, 8,9-, 11,12- and 14,15-EET) with [$^2H_8$]-AA used as internal standard and eluted at 36.40 min. All of the four regioisomers EET-PFB-esters were eluted at similar time and appeared as a major peak at 42.95 min. Attempts to separate them using columns with different polarities, such as HP5, HP50, CPsil, or RSL 500 and modulation of the temperature rise during the GC/MS run were unsuccessful. This confirms the inability of GC/MS

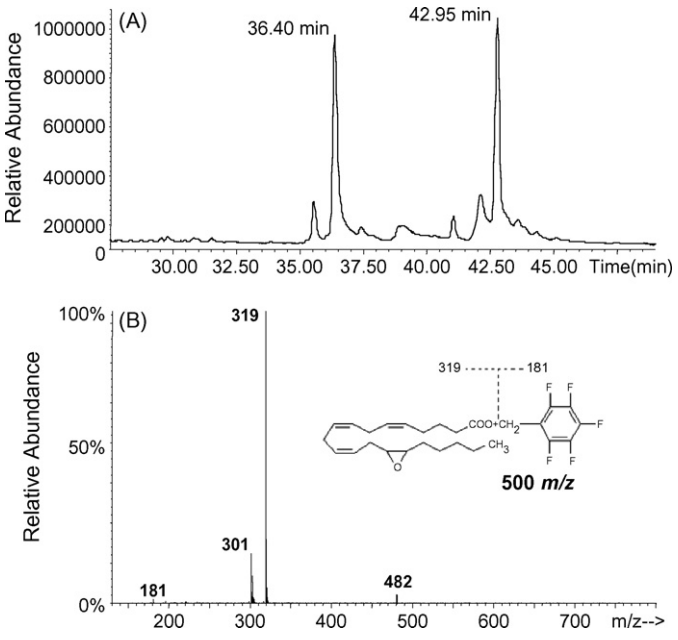

Fig. 1. (A) Gas chromatography profile of a mixture of the four regioisomers EETs (1 ng injected) and [$^2H_8$]-AA (internal standard, 1 ng injected) derivatized as pentafluorobenzyl esters (PFB-esters). This chromatogram represents the extracted ion of EETs (*m/z* 319) and [$^2H_8$]-AA (m/z 311) from a SIM acquisition. EET-PFB-esters and [$^2H_8$]-AA-PFB-ester were eluted at 42.95 and 36.40 min, respectively. (B) NICI mass spectrum of PFB-ester of 14,15-EET with methane as reagent gas. The characteristic peaks are a major ion fragment at *m/z* 319 corresponding to [M–H–PFB]$^-$ and a minor one at *m/z* 301 due to the subsequent loss of H_2O [M-H-PFB-H_2O]$^-$. The peak at *m/z* 319 for [M–H–PFB]$^-$ was monitored in the selected ion monitoring (SIM) mode.

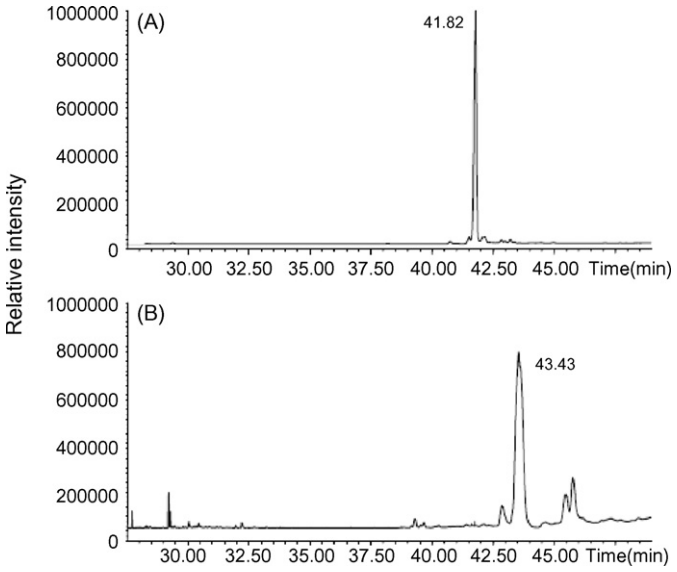

Fig. 2. Shift of EET retention time by addition of fatty acids in the medium: (A) The retention time for EETs alone (10 ng) in RBC hemolysate was 41.82 min. (B) Addition of arachidonic acid, eicospentaenoic acid and docosahexaenoic acid (10 μg) to EETs (10 ng) delayed the elution of EETs to 43.43 min. Chromatograms obtained by ion monitoring at *m/z* 319 (EETs).

to separate the monoepoxides of AA as previously reported [32]. In the same way, separation of the four EET-corresponding diols, i.e. 5,6-, 8,9- 11,12- and 14,15-DHET remains also very difficult [32]. In our chromatographic conditions only 14,15-DHET eluted separately from the other DHETs (data not shown). These attempts for EET or DHET regioisomer separation led to a rather high retention time of EETs (43 min) which can be divided by 2 (19 min) when the oven temperature is set at 60 °C for 5 min, then rised to 290 °C at the rate of 20 °C min^{-1} and held for 5 min at 290 °C (data not shown).

The NICI mass spectra of all regioisomers of EET-PFB-esters (M_r 500) exhibit the same spectra with a main ion at *m/z* 319 corresponding to $[M\text{-}PFB]^-$, a smaller ion at *m/z* 301 indicative of the subsequent loss of H_2O and two minor ions at *m/z* 181 and 482 corresponding to the $[C_7F_5H_2]^-$ and to the $[M–H_2O]^-$ fragments, respectively (Fig. 1B). $[^2H_8]$-EET-PFB-esters (M_r 508) have similar mass spectra to EET-PFB-esters with a major fragment at *m/z* 327 corresponding to the $[M–PFB]^-$ and a minor fragment at *m/z* 309 (subsequent loss of H_2O). $[^2H_8]$-AA was characterized as a PFB-ester by the major fragment at *m/z* 311 $[M–PFB]^-$. These NICI spectra of the four EETs are in agreement with those previously reported in the literature [32,40–42]. Formation of the PFB esters from free EETs, which is a very rapid reaction, has the advantage not only to strengthen the specificity and the sensitivity of the method [13,31,32] but also to protect EETs from further degradation during the time of analysis.

Analysis of biological samples revealed a shift of about 1 min in the elution of EETs when compared to standards. This was found to be attributable to a "matrix effect" likely induced by the release in large amounts of fatty acids from the phospholipids after hydrolysis of samples by PLA_2. Indeed, a similar shift was observed by addition of an overload of fatty acids in a RBC hemolysate (Fig. 2). One should note that because of the homology of structure and similarity of elution between 14,15-$[^2H_8]$-EET and EETs, the former was, at first, selected as internal standard. But, it had further to be replaced with $[^2H_8]$-AA because of the production by fragmentation, in NICI mode, of the same characteristic ion *m/z* 327, corresponding to the $[M\text{-}PFB]^-$ fragment, as docosahexaenoic acid, a fatty acid released by hydrolysis in large amounts in RBCs samples and eluted at the same time as 14,15-$[^2H_8]$-EET. $[^2H_8]$-AA was eluted slightly before AA and produced no interference with any other compound from the medium at *m/z* 311 monitoring.

Table 2
Calibration equations for EET standards.

Standard curve	Equation	Correlation coefficient
In RBC	$Y = 0.0104x + 0.0143$	0.994
In Plasma	$Y = 0{,}0111x - 0.0445$	0.996

A five-point calibration plot (50, 100, 150, 200, and 250 ng mL^{-1}) was drawn from a mixture of the four EETs added to either plasma or RBC hemolysate after SPE. The ratios of peak areas of the analytes/internal standard (250 ng mL^{-1}) were calculated and blanks (corresponding to endogenous EETs) were substracted. The calibration curves were drawn by linear regression analysis. EETs were monitored by the *m/z* 319.

3.1.1. Optimization of sample preparation

After extraction of RBC hemolysates, the phospholipids had to be separated from the chloroformic extract by chromatography on a SPE column because of the large amount of lipids present in the extract. The procedure involved 3 steps to, at first, eliminate the neutral lipids, then the free fatty acids and, at last, elute phospholipids. The removal of free fatty acid was monitored by using ^{14}C-AA or ^{14}C-EETs and showed recoveries of 95.23% and 85.92%, respectively (Table 1). The elution of phospholipids was then checked from RBCs samples: at each step, the elution phases were analyzed by GC/MS/NICI after addition of AA-d8 as internal standard and hydrolysis by PLA_2. When comparing the different SPE columns, i.e. Sep Pak C18 and Oasis® MCX, Bond Elut Jr, -C18, -C18+, -C18–Sax, and -C18-Aminopropyl, the proportion of EETs found in the fraction corresponding to the elution of phospholipids ranged from 50 to 93% (data not shown), the C18-aminopropyl displaying the highest recovery estimated at 93.46 ± 2.62% (Table 1). When the whole elution of phospholipids was checked by washing the SPE column with 4 mL MeOH again, the C18-Sax presented 10% of total EETs in this last fraction. These data led us to choose the C18-aminopropyl SPE column for sample purification as it was the best for the separation of phospholipids. Such a purification procedure was not required prior to the analysis of plasma samples and/or the determination of free EETs in RBCs, i.e. without hydrolysis. This underlines the need for specific protocols of purification for each tissue before GC/MS analysis.

3.1.2. Validation of the method

3.1.2.1. Calibration curves. To plot the standard curves, spiked amounts of 50–250 ng of EETs and 250 ng of internal standard ($[^2H_8]$-AA) were added to 1 mL of RBC hemolysate after SPE or

Table 1
SPE separation of human RBC phospholipids on C18-aminopropyl column.

Elution phase	% of ^{14}C-AA	% of ^{14}C-EETs	% of EETs from phospholipids
Chloroform/propanol (2:1)	2.88 ± 1.24	5.37 ± 0.44	1.76 ± 0.04
2% acetic acid in diethyl ether	95.23 ± 2.27	85.92 ± 0.29	4.78 ± 2.64
Methanol	1.89 ± 1.03	8.54 ± 0.18	93.46 ± 2.62

Results are expressed in percent of the total amounts disposed on the SPE column for ^{14}C-AA and ^{14}C-EETs, and in percent of the total EETs detected after phospholipid hydrolysis. Mean ± S.D. of 3 determinations.

Table 3
Intraday and interday precision.

Precision	EETs (ng mL^{-1})	CV (%)
Intraday	239.2 ± 7.9	3.29
	104.6 ± 4.3	4.11
interday	166.5 ± 8.4	5.03
	40.3 ± 3.0	7.44

Data represent mean ± S.D., $n = 10$.

Table 4
Accuracy of EETs determination in blood.

Surcharge of EETs added (ng)	Amounts found (ng)	Recovery %
20	20.45	102
100	105.14	105
200	210.08	105

The recovery of analytes was determined by adding different amounts of a standard mixture of all eicosanoids in RBC hemolysate after SPE and by comparing the amounts found after extraction. Results represent mean of three determinations.

plasma. Then, a linear relationship was established between the ratio of EETs and [$^2H_8$]-AA) peak areas and the concentration expressed as ng mL^{-1} (Table 2). The linearity of the method was confirmed over a range of 0–100 ng injected for each EET, corresponding to concentrations in the range 0–50 μg mL^{-1} (data not shown). All correlation coefficients r were over 0.994.

3.1.2.2. Limit of detection. The limits of qualitative and quantitative detections were found to be 6 pg (3 ng mL^{-1}) and 20 pg (10 ng mL^{-1}) of EETs, respectively, for "pure" standard samples while the limit of quantification in biological samples was 250 pg injected corresponding to a concentration of 12.5 ng mL^{-1} in the biological sample.

3.1.2.3. Precision. The intraday precision range was within 3.3% and 4.1% in RBC hemolysates; the interday variation was within 5 and 7.4% in RBC or plasma samples, respectively (Table 3). Values inferior to 10% indicated that the procedure related to plasma as to RBCs was reproducible.

3.1.2.4. Accuracy and recovery. The accuracy was checked by the determination of the relative recovery. The recovery yield for analytes was 104 ± 2% for added concentrations ranging from 20 to 200 ng mL^{-1} in biological samples (Table 4) indicating an accurate method for the concentrations measured.

3.1.3. Biological application

Among the EETs quantified in RBCs and in plasma before and after hydrolysis of phospholipids by PLA_2, the former were considered as free EETs, whereas the latter were assessed as total EETs. The finding of less than 3% of free EETs (data not shown) means that most of EETs were bound to the phospholipids. Fig. 3 illustrates the release of EETs by hydrolysis of RBC phospholipids. These data are in agreement with previous findings in the rat [4].

According to this study of EET concentrations in 10 healthy subjects, total EETs accounted for 106 ± 37 ng mL^{-1} in plasma and 33.4 ± 8.5 ng per 10^9 RBCs. When calculated per mL of blood, plasma and RBC EETs corresponded to 58 ± 20 and 182.5 ± 57 ng mL^{-1}, respectively, indicating that RBCs contain 3-fold more EETs than plasma (Fig. 4). However, the good correlation found in this study between RBCs and plasma values ($r = 0.95$, $p < 0.01$) suggests the existence of an equilibrium between both compartments. Moreover, one should note that these EETs plasma levels are in the same order of magnitude as those recently reported [29].

It is worth underlining that EET concentrations in blood samples are very unstable: they are decreased by 20–30% over the first 60 min following the withdrawal of blood, and this fall can reach 70–80% after 8 h at room temperature (data not shown). However, this process can be slowed down by storage of the blood samples at 4 °C. Though the main *in vivo* EET catabolic pathway is conversion to the corresponding DHETs by a soluble epoxide hydrolase (sEH) [6], no quantifiable amount of DHETs was detected by the

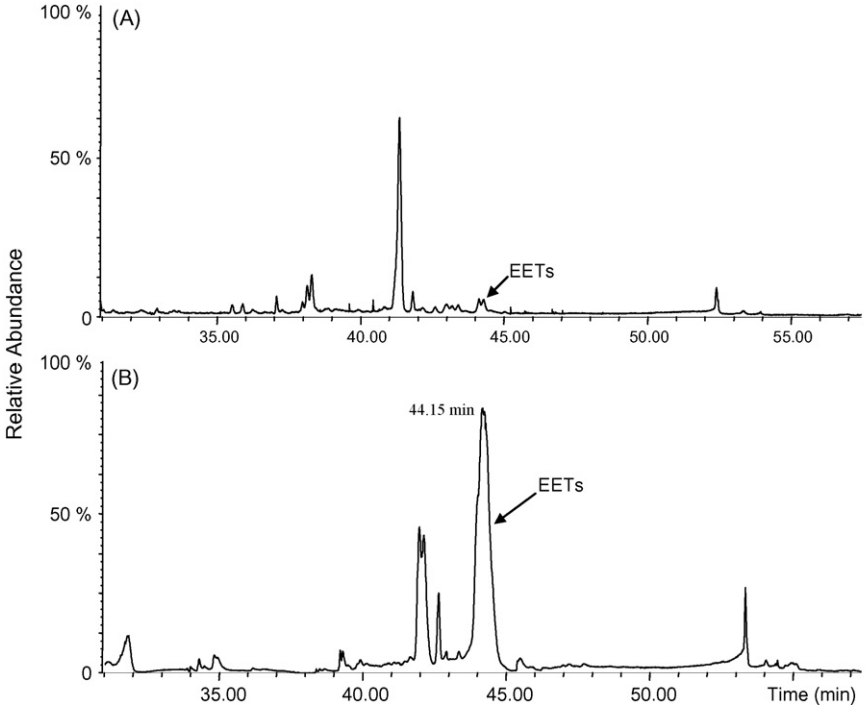

Fig. 3. Release of EETs from phospholipids following hydrolysis by *Naja Mossambica Mossambica* PLA_2 on human RBCs. (A) control; (B) PLA_2 10 units at 37 °C during 12 min in 1 mL tris buffer pH 8.9.

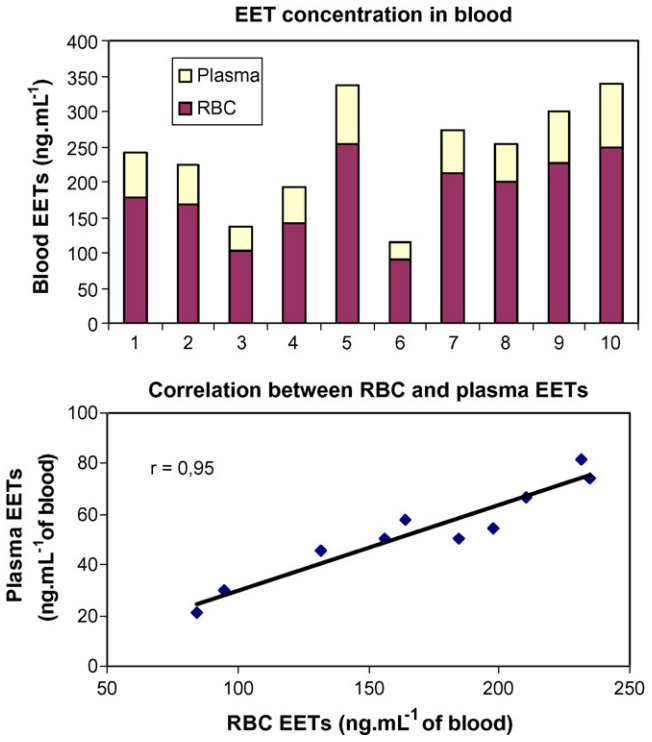

Fig. 4. Concentration of EETs in human blood: (A) EETs released from phospholipids of RBCs and plasma per milliliter of blood from healthy volunteers ($n = 10$). (B) Correlation between plasma and RBC EET levels.

proposed method despite the low limit of quantitative detection (30 ng mL^{-1}). This raises the question of the *in vitro* metabolism of EETs. Nevertheless conservation of the samples at 4 °C slowed EET decrease. Therefore, samples must be kept on ice and processed immediately.

4. Conclusion

In this paper, we have carefully described a sensitive and specific GC/MS/NICI method for EET determination in blood and quantified total EETs in red blood cells and plasma from 10 healthy volunteers. This method is suitable for routine analysis. Our data show that EETs levels in RBCs are 3 times those of plasma and that both values are well correlated. Thus, this methodology will be of valuable help to gain more insight into the role of endogenous EETs in pathologies such as hypertension, cardiovascular diseases or inflammation.

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