A method for the determination of 5,6-EET using the lactone as an intermediate in the formation of the diol

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Abstract The 5,6 epoxyeicosatrienoic acid (5,6-EET) exhibits a range of biological activities but the functional significance of this labile eicosanoid is unknown due, in part, to difficulties of quantitation in biological samples. We have developed a sensitive and specific method to measure 5,6-EET utilizing its selective capacity to form a lactone. The initial conversion of 5,6-EET and 5,6-dihydroxyeicosatrienoic acid (5,6-DHT) to 5,6-δ-lactone is followed by selective purification using reverse phase high performance liquid chromatography (HPLC), reconversion to 5,6-DHT and quantitation by gas chromatography-mass spectrometry (GCMS). In oxygenated Krebs' buffer, 5,6-EET degrades to 5,6-δ-lactone and 5,6-DHT with a $t_{1/2} \approx 8$ min. In the presence of camphorsulfonic acid, 5,6-EET and 5,6-DHT convert to a single HPLC peak ($\lambda = 205$) comigrating with 5,6-δ-lactone. Incubation of 5,6-δ-lactone with triethylamine resulted in a single HPLC peak with the retention time of 5,6-DHT. In the perfusate from the isolated kidney, release of 5,6-EET (20 ± 5 pg/ml), measured indirectly via conversion to 5,6-DHT, was approx. 6-fold less than that reported for prostaglandin E_2 (PGE_2) and 20-HETE. The coronary perfusate concentration of 5,6 EET was 9 ± 2 pg/ml. 5,6-EET recovered from renal and coronary perfusates was increased 2-fold to 45.5 ± 5.5 pg/ml and 21.6 ± 6.3 pg/ml, respectively, by arachidonic acid.—Fulton, D., J. R. Falck, J. C. McGiff, M. A. Carroll, and J. Quilley. **A method for the determination of 5,6-EET using the lactone as an intermediate in the formation of the diol. *J. Lipid Res.* 1998.** 39: **1713–1721.**

Supplementary key words 5,6-EET • 5,6-DHT • 5,6-δ-lactone • GC–MS • heart • kidney

Cytochrome P450-dependent (P450) metabolism of arachidonic acid (AA), the so-called third pathway, yields HETEs via ω- and ω-1 hydroxylases and epoxides via epoxygenases (1). These eicosanoids exhibit a range of biological activities affecting vascular and renal function and, therefore, contribute importantly to the regulation of fluid volume and blood pressure (1). Thus, they are vasodilator (2–6) or vasoconstrictor (7–10), presumably through modulation of ion channels (11–14); they also influence renal tubular function by modifying the activity of ion transport systems (15–18). P450 AA products have been implicated in the autoregulation of renal blood flow (19, 20), myogenic responses (21, 22), and mediation/modulation of vasoactive hormones including angiotensin II (17, 23), vasopressin (24), and bradykinin (25, 26). Moreover, 20-HETE has been implicated in the development of hypertension in some rat models (27–29) and in the hemodynamic adaptations to cirrhosis (30). EETs may play a role in the adaptation to high salt intake (31, 32) preventing elevation of blood pressure when the animal is challenged with high salt. There is now considerable evidence favoring identification of one or more of the EETs as endothelium-derived hyperpolarizing factor(s) (EDHF) (33–35).

Most studies addressing the functional role of P450-derived eicosanoids have relied on the use of inhibitors of P450, the specificity of which has been questioned. Very few studies have been able to correlate measurements of P450-derived eicosanoids with functional changes. This is particularly true for the epoxides that exist as four regioisomers, each of which has two stereoisomeric configurations. Three of the epoxides, the 8,9-, the 11,12-, and the 14,15-EET are relatively stable and can be measured directly (31) or after their conversion to the respective diols (31) once they are separated by high performance liquid chromatography (HPLC). However, it is difficult to separate the epoxide regioisomers using reverse phase HPLC. For 5,6-EET there are additional difficulties that have precluded an accurate estimate of its formation. First, 5,6-EET is unstable with a half life of approximately 8 min in physiological buffer and, therefore, does not lend itself to direct chemical measurement. Secondly, 5,6-EET spontaneously and, perhaps, enzymically degrades to the diol and lactone (36) (**Fig. 1**). As a result, measurements of 5,6-EET have not been attempted in most studies. Mea-

Abbreviations: EET, epoxyeicosatrienoic acid; DHT, dihydroxyeicosatrienoic acid; P450, cytochrome P450; AA, arachidonic acid; HPLC, high performance liquid chromatography; CG–MS, gas chromatography–mass spectrometry; PGE_2, prostaglandin E_2; EDHF, endothelium-derived hyperpolarizing factor.

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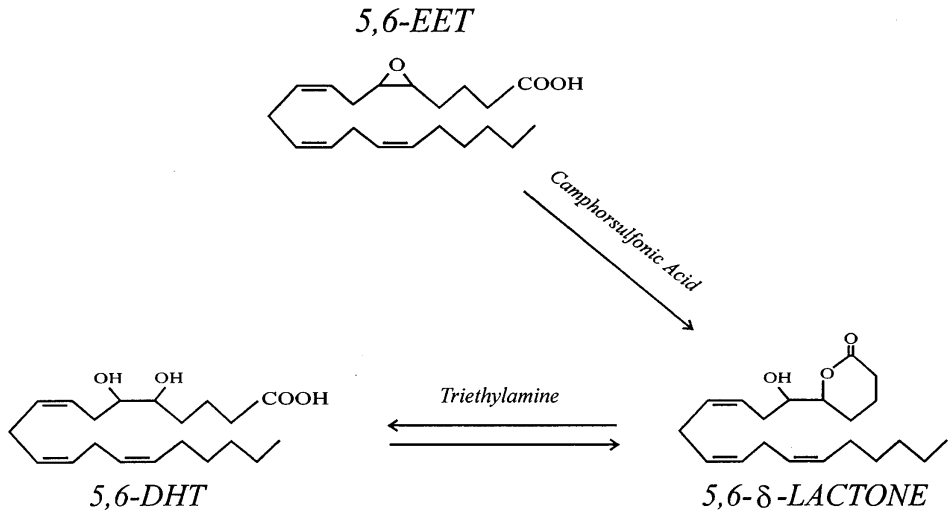

Fig. 1. Metabolism of 5,6-EET. Schematic representation of 5,6-EET and metabolites, 5,6-δ-lactone and 5,6-DHT.

surement of the diol, which has been used as an index of 5,6-EET formation by Makita et al. (32) may not be an accurate reflection of 5,6-EET release because of the simultaneous formation of the 5,6-δ-lactone. Thus, measurements of the diol alone may underestimate the 5,6-EET. However, measurements of the lactone as described by Yamane and Abe (37) are a valid alternative provided both the EET and the diol are fully transformed and the lactone is not reconverted to the diol.

We directed our attention to the problem of measuring 5,6-EET because of our interest in P450-derived eicosanoids as potential mediators of the NO-independent vasodilator actions of bradykinin which are dependent on activation of phospholipases and potassium channels and are attenuated by inhibitors of P450 (38). Our studies using the rat heart have revealed only the release of EETs, not HETEs, into perfusates analyzed by gas chromatography–mass spectrometry (GC–MS). Based on recent results, only 5,6-EET, of the EETs, is a viable candidate as the EDHF mediating the response to bradykinin in the rat heart and kidney (38). Here we report a method for measuring 5,6-EET. Our approach was to use the unique property of 5,6-EET to form a δ-lactone. Thus, 5,6-EET and its diol were converted to the δ-lactone which was separated from other EETs via reverse phase HPLC. The purified δ-lactone was then converted to the diol for estimation by GC–MS, an established method. Thus, the very property of 5,6-EET to spontaneously degrade, which has been a hindrance to its quantitation, can be used to advantage, i.e., the selective ability of 5,6-EET, but not the other EETs, to form a lactone provides the basis for a simple method to measure this eicosanoid.

METHODS

Degradation of 5,6-EET

5,6-EET (3 μg) was incubated in oxygenated (95% O_2/5% CO_2) Krebs' buffer (millimolar composition: NaCl 118, $NaHCO_3$ 25, $CaCl_2$ 1.9, $MgSO_4$ 1.19, KCl 4.75, KH_2PO_4 1.19, and dextrose 11.1), pH 7.4, at 37°C for various time periods to assess the temporal transformation of 5,6-EET to DHT and δ-lactone. The Krebs' buffer was acidified to pH 4.0 and 3 volumes of ethyl acetate were used to extract 5,6-EET and resulting products. The organic phase was decanted, dried with a Savant Speed Vac (SVC 100, Farmingdale, NY) and subjected to HPLC analysis. Reverse phase HPLC was performed using a C18 μBondpack column (4.6 mm × 24 mm, Waters Associates) and a linear gradient of 1.25%/min from acetonitrile–H_2O–acetic acid 50:50:0.1 to acetonitrile at a flow rate of 1 ml/min. Metabolites were monitored using UV absorption at λ = 205 nM.

Conversion of EET, DHT to δ-lactone

The conversion of 5,6-EET and 5,6-DHT to a δ-lactone intermediate with a distinct HPLC retention time is a property not shared by other epoxide isomers and enables selective purification of 5,6-EET and metabolites. 5,6-EET (5 μg), 5,6-DHT(5 μg), and a mixture of 5,6-EET, DHT and 5,6-δ-lactone (3 μg) were suspended in chloroform that had previously been dehydrated with molecular sieves. Camphorsulfonic acid (25 μg) was added to this solution and the reaction was left to proceed at room temperature for 40 min. After acidification, the pH was adjusted to 7.0 with 4.2% $NaHCO_3$ and the organic phase was extracted with chloroform–methanol–water 2:1:1. The organic phase was dried using a Savant Speed Vac, resuspended in 20 μL of methanol and injected onto the HPLC. HPLC analysis was performed as described above.

Conversion of δ-lactone to DHT

The ability to transform 5,6-δ-lactone back to 5,6-DHT allows for efficient derivatization and sensitive GC–MS without need for further purification. 5,6-δ-lactone (5 μg) was suspended in 100 μL methanol, 100 μL H_20, and 5 μL triethylamine and incubated at 37°C for 1 h. The reaction mixture was then dried directly in the Savant Speed Vac and analyzed by HPLC as described above.

5,6-δ-lactone & EET standards

Deuterium-labeled 5,6-δ-lactone was prepared according to the method of Corey, Niva, and Falck (39) with the following modifications. d8-Arachidonic acid (10 mg) and 0.5 μCi of 1-^{14}C-

labeled arachidonic acid were reacted with potassium triiodide (8 eq) in the presence of potassium bicarbonate (5 eq) in tetrahydrofuran–water 2:1 overnight at 4°C. Excess iodine was removed with a saturated solution of sodium sulfite and the intermediate iodolactone was extracted with hexane and reacted with lithium hydroxide solution (0.2 N) in tetrahydrofuran (1:2) v/v for 3 h at room temperature. The resultant 5,6-EET was extracted with ethyl acetate, exposed to H_2O for 4 h, and the δ-lactone of 5,6-EET was purified to a single peak by reverse phase HPLC as described above. High specific activity 5,6-EET ([^{3}H]-arachidonic acid; 221 Ci/mmol) was synthesized as above without exposure to H_2O.

Standard curve

Deuterium-labeled 5,6-δ-lactone (30 ng) was incubated with varying amounts of standard 5,6-δ-lactone (100 ng–200 pg) and converted to 5,6-DHT as described above. Pentafluorobenzyl esters of 5,6-DHT (d0/d8), dissolved in acetonitrile (100 μL), were prepared by the addition of pentafluorobenzylbromide (5 μL) and N,N-diisopropylethylamine (5 μL) and the derivatization was continued at room temperature for 30 min. Samples were dried and trimethylsilyl (TMS) ethers of hydroxyls were prepared by suspending samples in N,O-bis(trimethylsilyl)trifluoroacetamide (80 μL) and pyridine (20 μL) for 30 min at room temperature. After drying, samples were suspended in 50 μL isooctane and 1-μL aliquots were injected into a GC (HP5890) column (DB-1; 15.0 m; 0.25 mm; inner diameter 0.25 μm film thickness Supelco) using a temperature program ranging from 180 to 300°C at a rate of 25°C/min. Methane was used as a reagent gas at a flow resulting in a source pressure of 1.3 torr, and the mass spectrometer (Hewlett-Packard 5989A) was operated in the negative ion chemical ionization mode (NCI, electron capture). Standard 5,6-DHT (d0) was monitored at ion *m/z* 481 and deuterium (d8)-labeled 5,6-DHT at *m/z* 489. A standard curve was constructed from ratios of peak area, determined by integration, of d0 and d8 5,6-DHT.

Biological samples

Male Wistar rats weighing approximately 350–450 g were anesthetized with pentobarbital (65 mg/kg, IP) and kidneys and hearts were perfused as described previously (25, 26). Briefly, after a midline laparotomy and administration of heparin (1000 U/kg) the right kidney was cannulated via the mesenteric artery and perfused at constant flow, approx. 11 ml/min. (Watson-Marlow, model 502S, New Brunswick Scientific, Edison, NJ) with oxygenated Krebs' buffer maintained at 37°C. The right kidney was excised to permit collection of perfusate. After isolation of the kidney, a thoractomy was performed and the heart with attached aorta was excised, flushed of blood, and immersed in ice-cold saline. The aorta was cannulated and the heart was perfused at constant flow, approx. 10 ml/min, with Krebs' buffer. After cannulation, the kidney was treated with nitroarginine (50 μm) and phenylephrine ($2–4 \times 10^{-7}$ m) to elevate perfusion pressure from 75–90 mmHg to approximately 200 mm Hg. The coronary vasculature was preconstricted with nitroarginine (50 μm) to elevate perfusion pressure from 30–40 mm Hg to approximately 135 mm Hg. Once a stable elevated perfusion pressure was obtained, perfusates were collected (control; 15 min) prior to addition of arachidonic acid (0.5 μg/ml; 15 min). Indomethacin (2.8 μm) was included in all perfusates to eliminate the potential metabolism of 5,6-EET by cyclooxygenase. Internal standard (5,6-δ-lactone-d8, 18 ng) was added to perfusates prior to acidification (pH 4.0) and extracted with ethyl acetate (3 volumes). We chose 5,6-δ-lactone over 5,6-EET as the internal standard because of its stability. As 5,6-EET spontaneously degrades to the lactone and DHT, the use of 5,6-EET as the internal standard results in unknown proportions of 5,6-EET, DHT, and lactone being added to the samples. The organic phase was decanted and evaporated using a Brinkmann rotary evaporator (Westbury, NY). Samples were then resuspended in 1 ml of chloroform that had previously been treated with molecular sieves to remove H_2O and acidified with camphorsulfonic acid (25 μg) for 40 min, conditions previously determined to be optimal for converting both 5,6-EET and 5,6-DHT to 5,6-δ-lactone. Samples were adjusted to pH 7.0 with 4.2% $NaHCO_3$ and the organic phase was extracted from chloroform–methanol–water 2:1:1, evaporated, and the residues were subjected to reverse phase HPLC as described above. Fractions containing 5,6-δ-lactone were collected, based on known retention times of authentic 5,6-δ-lactone, and evaporated using the Savant Speed Vac. These samples were resuspended in 100 μL methanol, 100 μL H_2O, and 5 μL triethylamine and incubated at 37°C for 1 h. After conversion to 5,6-DHT, samples were again dried using the Savant, resuspended in 100 μL acetonitrile, and derivatized as described above. Samples were suspended in 50 μL isooctane and 1-μL aliquots were injected into the GC as described above. The endogenous 5,6-DHT was identified (ion *m/z* 481) by comparison of GC retention times with authentic 5,6-DHT and quantitated by calculating the ratio of abundance with d8-5,6-DHT (*m/z* 489).

Materials

Arachidonic acid (d8), molecular sieves (1/16 inch pellets, 4Å pore diameter), bis(trimethylsilyl)trifluoroacetamide, pyridine, triethylamine (TEA), indomethacin, nitroarginine, and camphorsulfonic acid were obtained from Sigma. Pentafluorobenzyl bromide and N,N-diisopropylethylamine were from Aldrich. [1-^{14}C] and [^{3}H]arachidonic acid were from New England Nuclear. Sodium arachidonate was obtained from NuChek Prep, Elysian, MN. 5,6-EET, 5,6-DHT, 5,6-δ-lactone, LTB_4, and 5S,6R-diHETE were all purchased from Cayman Chemical. All solvents were of HPLC grade.

RESULTS

Degradation of 5,6-EET and 5,6-δ-lactone

The time-dependent lability of 5,6-EET was determined after incubations of standard 5,6-EET in oxygenated Krebs' buffer maintained at 37°C and analyzed by reverse phase HPLC with UV detection (λ = 205, **Fig. 2 A–E**). Within 30 min over 90% of 5,6-EET (retention time (RT) ≅ 16.5 min) had degraded to a product eluting at the same retention time as authentic 5,6-δ-lactone (RT ≅14 min). 5,6-EET had an approximate half life of 8 min. Incubation periods longer than 30 min revealed that in oxygenated Krebs' buffer, formation of 5,6-DHT (RT ≅ 9.5 min) is not derived from 5,6-EET but from 5,6-δ-lactone (Fig. 2E).

The conversion of 5,6-δ-lactone to 5,6-DHT in Krebs' at 37°C required longer incubation times than conversion of 5,6-EET to 5,6-δ-lactone (**Fig. 3 A–E**). As detected by reverse phase HPLC (λ = 205), standard 5,6-δ-lactone spontaneously converted to 5,6-DHT with a half life of approximately 150 min (Fig. 3 A–E). 5,6-DHT is relatively stable in oxygenated Krebs' and was not significantly transformed within 10 h (Fig. 3E).

Chemical conversion of 5,6-EET and DHT to δ-lactone

Incubation of 5,6-EET with camphorsulfonic acid resulted in the conversion of 5,6-EET within 40 min to a

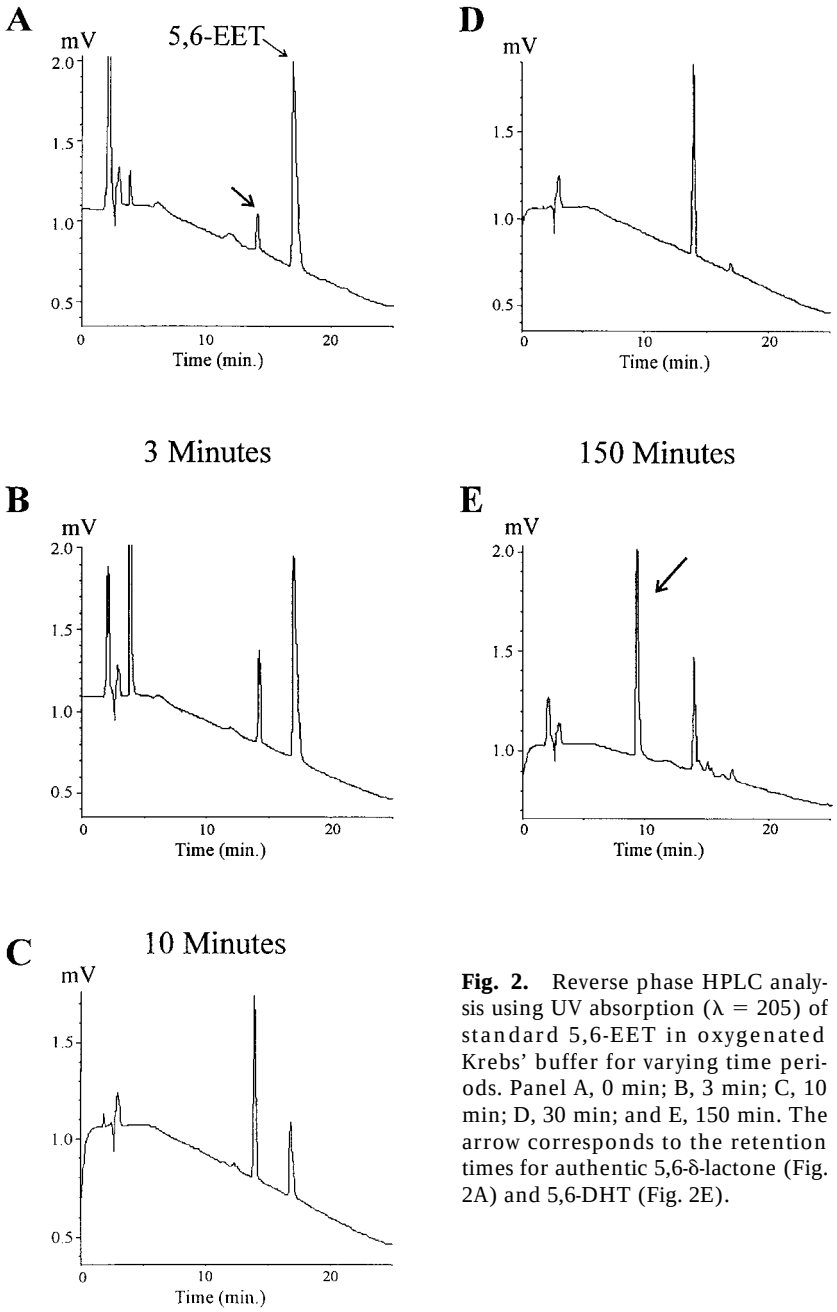

Fig. 2. Reverse phase HPLC analysis using UV absorption (λ = 205) of standard 5,6-EET in oxygenated Krebs' buffer for varying time periods. Panel A, 0 min; B, 3 min; C, 10 min; D, 30 min; and E, 150 min. The arrow corresponds to the retention times for authentic 5,6-δ-lactone (Fig. 2A) and 5,6-DHT (Fig. 2E).

product co-migrating with authentic 5,6-δ-lactone (**Fig. 4 A–B**). Within the same period of time, 5,6-DHT was also converted, in the presence of the acid, to a single peak eluting with the same retention time as 5,6-δ-lactone (Fig. 4C–D). Conversion of 5,6-DHT was complete within 30 min, whereas only 90% of 5,6-EET was converted to 5,6-δ-lactone during the same time frame (data not shown). The treatment of a mixture of equal amounts of 5,6-δ-lactone, 5,6-DHT, and 5,6-EET (**Fig. 5A**) for 40 min with camphorsulfonic acid also resulted in the appearance of a single peak with the same retention time as 5,6-δ-lactone (Fig. 5B).

Standard 5,6-δ-lactone, when exposed to TEA in the presence of methanol and water for 60 min, produced a single HPLC product comigrating with authentic 5,6-DHT (Fig. 5C–D).

Epoxide regioisomers 8,9-EET (RT ≅ 15.5 min), 11,12-EET (RT ≅ 15.4 min) and 14,15-EET (RT ≅ 14.5 min) were not modified after incubation with camphorsulfonic acid or triethylamine (data not shown). Eicosanoids with structures similar to 5,6-DHT, LTB_4 (RT ≅ 6 min), and 5S,6R-diHETE (RT ≅ 6 min) when incubated for 40 min in the presence of camphorsulfonic acid produced less polar metabolites (≅30–50% conversion) that had retention times of 11 and 8.5 min, respectively (data not shown).

Standard curve

Figure 6 shows a standard curve derived from varying amounts of 5,6-δ-lactone standard and a constant amount of deuterium labeled internal standard. The ratio of d0/

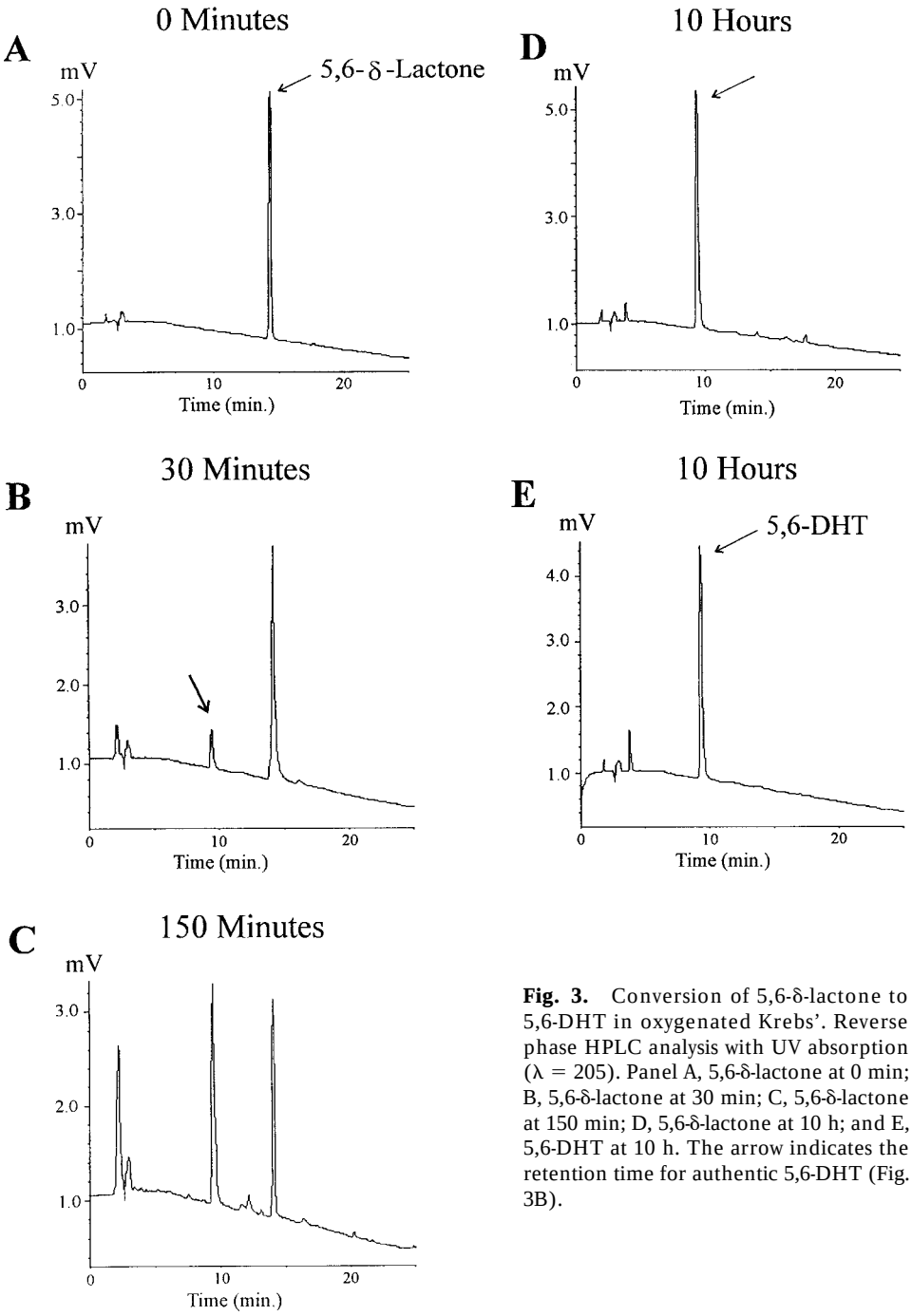

Fig. 3. Conversion of 5,6-δ-lactone to 5,6-DHT in oxygenated Krebs'. Reverse phase HPLC analysis with UV absorption (λ = 205). Panel A, 5,6-δ-lactone at 0 min; B, 5,6-δ-lactone at 30 min; C, 5,6-δ-lactone at 150 min; D, 5,6-δ-lactone at 10 h; and E, 5,6-DHT at 10 h. The arrow indicates the retention time for authentic 5,6-DHT (Fig. 3B).

d8 5,6-DHT was linear from 100 ng to 200 pg with a correlation coefficient of 0.997.

Biological samples

The standard 5,6-δ-lactone that was used as an internal standard was purified to homogeneity by HPLC and GC–MS analysis determined that >99% was d8. Extraction efficiencies for ≅20 pg of [^{3}H]5,6-EET, -DHT, and δ-lactone from acidified Krebs' buffer with 3 volumes of ethyl acetate were >95% for 5,6-EET and 5,6-δ-lactone and >70% for 5,6-DHT. The yield from ≅80 pg [^{3}H]5,6-EET after extraction from Krebs' buffer after conversion to δ-lactone and HPLC purification was 77%.

In isolated perfused kidneys, phenylephrine increased perfusion pressure to approximately 200 mm Hg from a basal perfusion pressure of 75–90 mm Hg at a flow of 11.3 ± 1.3 ml/min. Nitroarginine increased coronary perfusion pressure from 35 mm Hg to 140 mm Hg in hearts perfused at 10.0 ± 0.1 ml/min. Under control conditions, 20.3 ± 4.5 pg/ml of 5,6-DHT was recovered from the renal effluent and 8.7 ± 2.1 pg/ml in perfusate from the rat coronary circulation. Arachidonic acid (0.5 μg/ml) increased the amount of 5,6-DHT recovered ≅2 to 2.5-fold to 45.54 ± 5.5 pg/ml in the kidney and 21.6 ± 6.3 pg/ml in the heart (**Fig. 7**). Figure 7 (inset) shows a typical GC–MS chromatogram of 5,6-DHT (RT ≅ 4.98 min) recovered from renal

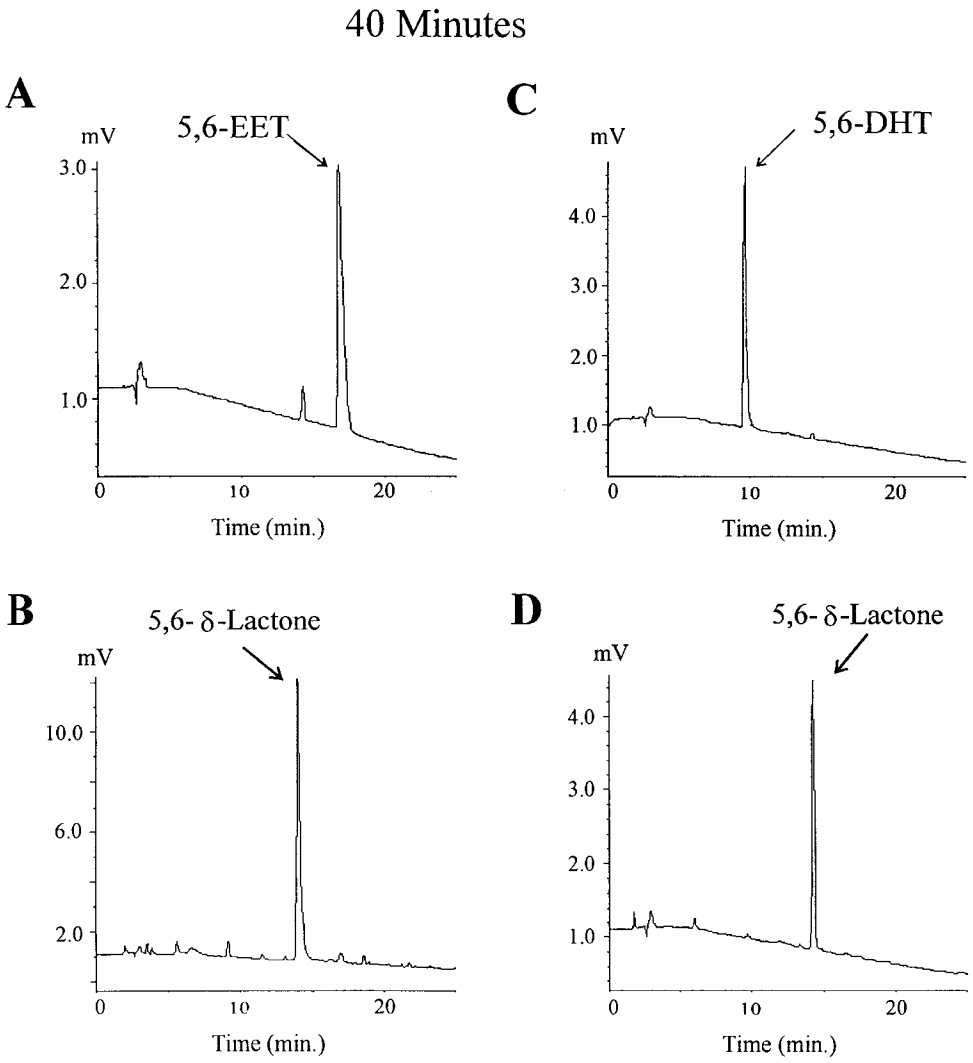

Fig. 4. Conversion of 5,6-EET and 5,6-DHT to 5,6-δ-lactone. Reverse phase HPLC chromatogram using UV detection at λ = 205. Panel A, Standard 5,6-EET; B, 5,6-EET after incubation with camphorsulfonic acid for 40 min; C, standard 5,6-DHT; and D, 5,6-DHT after incubation with camphorsulfonic acid for 40 min. The peak in Fig. 4B and D correspond to the HPLC elution time for authentic 5,6-δ-lactone.

perfusates under basal conditions and after arachidonic acid stimulation. Based on these results, we could detect as low as 15 pg 5,6-DHT injected into the GC–MS.

DISCUSSION

In oxygenated Krebs' buffer at 37°C, 5,6-EET spontaneously degrades to 5,6-δ-lactone and 5,6-DHT with an approximate half life of 8 min. Further, 5,6-δ-lactone undergoes transformation and, ultimately, is completely converted to 5,6-DHT. Because of the relatively rapid spontaneous breakdown and perhaps enzymatic conversion of 5,6-EET (36), a mixture of epoxide, diol, and δ-lactone will likely be present at any given time and thus, measurement of 5,6-EET or either of its metabolites will not accurately reflect the amount of 5,6-EET released into biological fluids. Indeed, the lack of an accurate method to measure 5,6-EET concentrations in biological fluids has been an obstacle in studies investigating the role of this eicosanoid. Therefore, we have developed a sensitive and selective method to quantitate total 5,6-EET in a biological specimen via a lactone intermediate and measurement of 5,6-DHT as an index of total 5,6-EET release. Our approach was to first convert 5,6-EET and 5,6-DHT to 5,6-δ-lactone which, following purification by HPLC, was converted back to the DHT for GC–MS analysis. Yamane and Abe (37) used a similar approach for estimating 5,6-EET in tissue homogenates by direct measurement of the lactone which they reported as the major degradation product during the incubation and extraction procedures. This, of course, is a viable alternative if the conversion of both 5,6-EET and its diol to the lactone is complete. If it is not or varies with the initial amount of the 5,6-EET or diol, then measurements of the lactone alone will be of limited accuracy.

For the method described here, the measurement of 5,6-EET from aqueous solutions involves a two-step conversion process. After extraction with ethyl acetate, 5,6-

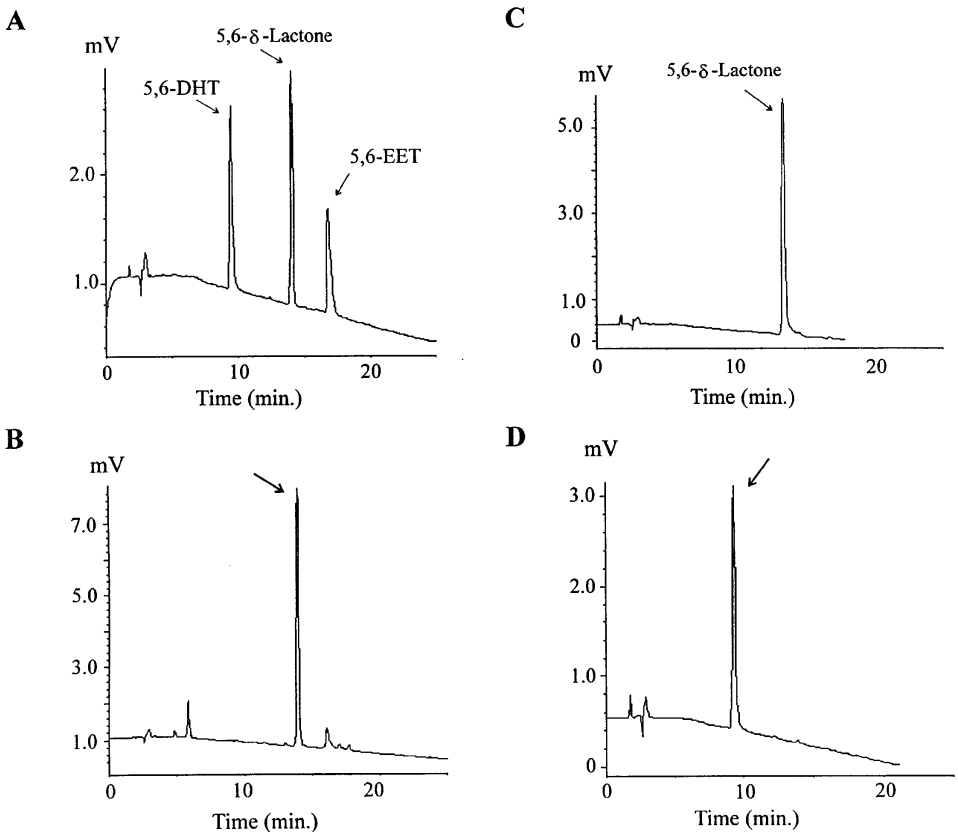

Fig. 5. Conversion of a mixture of 5,6-EET, 5,6-DHT, and 5,6-δ-lactone to 5,6-δ-lactone (A, B) and reconversion of 5,6-δ-lactone to 5,6-DHT (C, D). Reverse phase HPLC chromatogram (λ = 205) of 5,6-EET, 5,6-DHT and 5,6-δ-lactone prior to (A) and after (B) incubation with camphorsulfonic acid. The arrow signifies the retention time for standard 5,6-δ-lactone (Fig. 5B). HPLC chromatogram of standard 5,6-δ-lactone after incubation with triethylamine, methanol, and water for 1 . Panel C, standard 5,6-δ-lactone and D, 5,6-δ-lactone after treatment with triethylamine. The arrow indicates the retention time for authentic 5,6-δ-lactone (Fig. 5D).

EET and its products are converted to a single compound, 5,6-δ-lactone, via acidification in dehydrated chloroform. This is a rapid process as a mixture of epoxide, diol, and δ-lactone, the most likely constituents arising from the release of 5,6-EET in physiological solutions, was converted to a single peak, corresponding to 5,6-δ-lactone, within 45 min. The acid conversion is specific for 5,6-EET as the other epoxide regioisomers were unaffected within 45 min. However, structurally related eicosanoids, LTB_4 and the diol of LTA_4, 5S,6R-diHETE, were partially (30–50%) converted to a less polar product, presumably a δ-lactone, but this was well separated from 5,6-δ-lactone on reverse phase HPLC. As the retention time for 5,6-δ-lactone is distinct from the other epoxides and their respective diols, the HPLC fraction containing the δ-lactone was isolated and subjected to a second step, conversion to 5,6-DHT via treatment with the organic base, TEA, a reaction accelerated with H_2O. This process was complete within 1 h, as determined by the conversion to a single HPLC peak corresponding to the retention time for 5,6-DHT. The other EET regioisomers, 8,9EET, 11,12EET, and 14,15EET, were

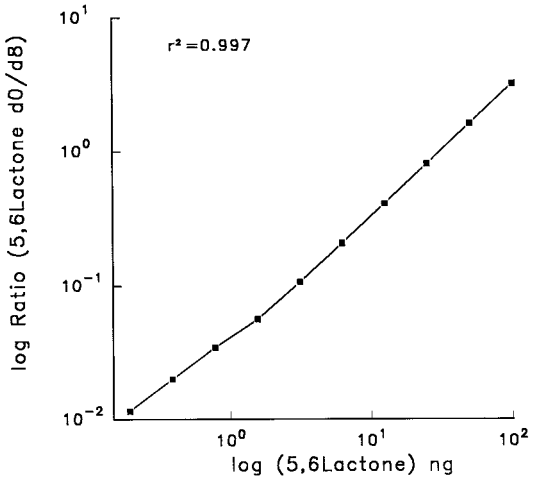

Fig. 6. Standard curve for 5,6-DHT. Serial dilutions of 5,6-δ-lactone (d0, 100 ng–200 pg) and 30 ng of 5,6-δ-lactone (d8) were converted to 5,6-DHT, derivatized, and subjected to single ion monitoring GC–MS analysis. Respective areas under the curve were integrated and a standard curve was derived from the ratio of d0/d8.

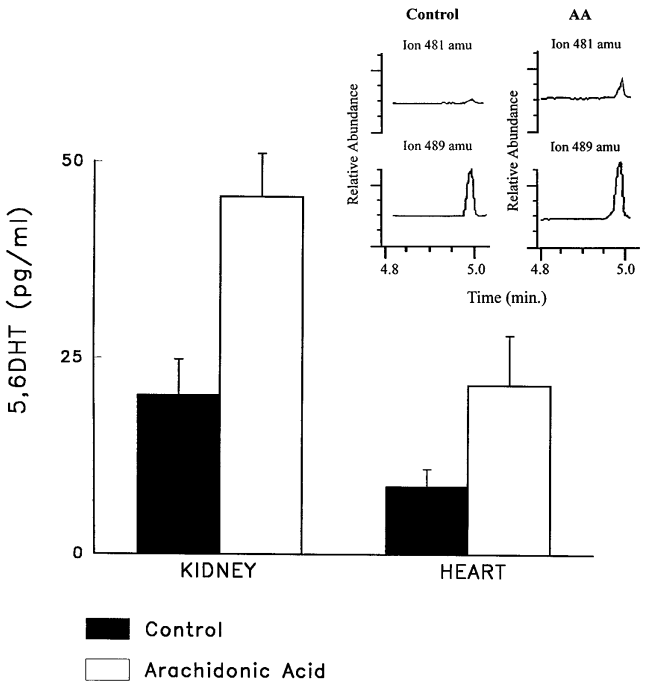

Fig. 7. Measurement of 5,6-DHT. GC–MS measurement of 5,6-DHT as an index of 5,6-EET release into the effluents of perfused rat kidneys (n = 3) and hearts (n = 4) after conversion to a δ-lactone intermediate, purification, and final conversion to 5,6-DHT. Inset, typical GC–MS chromatograms of renal perfusate prior to and after addition of arachidonic acid. Ion 481 and 489 amu refer to endogenous 5,6-DHT (d0) and internal standard 5,6-DHT (d8), respectively. The values for 5,6-DHT represent the amounts obtained from 15 min collection periods before and after addition of arachidonic acid (0.5 μg/ml) to the perfusate.

not modified by TEA. The conversion of 5,6-δ-lactone to 5,6-DHT permitted convenient derivatization without need for further purification.

This methodology was subsequently applied to biological samples obtained from isolated perfused kidneys and hearts treated with nitroarginine and indomethacin. Administration of arachidonic acid resulted in a 2- to 2.5-fold increase in 5,6-DHT from both kidneys and hearts. However, it remains to be determined whether this increase is a function of enzymatic synthesis, displacement of esterified 5,6-EET or its metabolites in membrane phospholipids, or a result of autooxidation of arachidonic acid. These issues can be readily addressed in future studies using inhibitors of arachidonate metabolism. The release of 5,6-EET from the kidney under basal conditions is approximately 6-fold less than we have previously reported for prostaglandin E_2 (40) and 20-HETE (10) and, after challenge with AA, the increase in release is less than that seen for prostaglandin E_2 (40). However, 5,6-EET represents only one of four EET regioisomers and in the rat kidney is not the predominant EET regioisomer (31). Consequently, release of total EETs from the perfused kidney is likely to be comparable to the release of 20-HETE. In the heart, the release of 5,6-EET is approximately 10- to 50-times less than the release of 6-keto $PGF_{1\alpha}$ under basal conditions (41, 42) and the fold increase after challenge with AA was also less (42). However, the relatively low quantities of 5,6-EET released from perfused organ systems may reflect rapid reacylation of this eicosanoid which may be the limiting factor, as opposed to the lability of this compound, in obtaining meaningful measurements.

In conclusion we have developed a selective and sensitive method for estimation of 5,6-EET that can be applied to the effluent of perfused organs. This method utilized the unique property of 5,6-EET and its DHT to undergo total conversion to the δ-lactone, providing an easy means to effectively separate 5,6-EET from other EET and DHT regioisomers. Reconversion of the δ-lactone to the corresponding DHT provides a stable compound that can be measured using GC–MS. We have utilized this methodology to measure 5,6-EET release from renal and cardiac perfusates.

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