Quantification of 20-hydroxyeicosatetraenoic acid by colorimetric competitive enzyme linked immunosorbent assay

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Analysis of 20-hydroxyeicosatetraenoic acid (20-HETE), a potent vasoconstrictor produced by the cytochrome P450 pathway, presently requires high-performance liquid chromatography (HPLC) and gas chromatography/ mass spectrometry (GC/MS). To simplify 20-HETE analysis, competitive ELISAs were developed using polyclonal anti-20-HETE coated ELISA plates to which free 20-HETE and 20-HETE conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP) were added. Assays were developed with and without a proprietary enhancer solution which allows for the extraction-free measurement of 20-HETE in urine samples. The bound 20-HETE-HRP or 20-HETE-AP was detected using 3,3′,5,5,′-tetramethylbenzidine and p-nitrophenyl phosphate, respectively. Sensitivities expressed as 80% B/B0, were 0·1 ng/ml for the HRP assay, and 0·5 ng/ml for the AP assay, with $r^2 = 0{\cdot}99$ for both formats. Of the 17 lipids tested for cross-reactivity, arachidonic acid showed the highest (0·32%) followed by racemic 5-HETE (0·07%) and 8,9-dihydroxyeicosatrienoic acid (DHET) (0·04%). Preliminary validation experiments examining serum and urine concentrations of 20-HETE yield values that fall within the ranges established by GC/MS in the literature. These ELISAs provide simple and inexpensive methods for the analysis of 20-HETE in biological samples.

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1. Introduction

20-Hydroxyeicosatetraenoic acid (20-HETE) is considered to be a critical vasoconstrictor in vascular and tubular mechanisms essential to the regulation of renal hemodynamics and extracellular fluid volume. In a recent comprehensive review, Richard Roman discussed the cytochrome P450 (CYP) metabolites of arachidonic acid primarily 20-HETE and epoxyeicosatrienoics (EETs) which stimulate vascular function by vasoconstriction and vasodilation, respectively, in kidney, liver, brain, vasculature, lung, pancreas, gastrointestinal tract, and other tissues (Roman 2002). More than 500 CYP genes have been identified and categorized by sequence homology. These are subdivided into 78 families, fourteen of which are expressed in mammalian tissue. Both 20-HETE and

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Abbreviations used: AP, Alkaline phosphatase; ANG II, angiotensin II; BCA, bicinchoninic acid; BSA, bovine serum albumin; CO, carbon monoxide; CYP, cytochrome P450; DCC, N′N-dicyclohexylcarbodiimide; DHET, dihydroxyeicosatrienoic acid; DMF, dimethylformamide; EET, epoxyeicosatrienoic acid; ELISA, enzyme linked immunosorbent assay; GC/MS, gas chromatography/mass spectroscopy; 20-HETE 20-hydroxyeicosatetraenoic acid; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; IgG, immunoglobulin G; NHS, N-hydroxysuccinimide; NO, nitric oxide; PBS, phosphate buffered saline (Dulbecco's, Ca^{+2}- and Mg^{+2}-free); PMA, phosphomolybdic acid; pNPP, p-nitrophenyl phosphate; SAS, saturated ammonium sulphate; Tg, thyroglobulin (porcine); TMB, 3,3′,5,5′,-tetramethylbenzidine; VSM, vascular smooth muscle.

EETs are produced in vascular smooth muscle (VSM). EETs act as vasodilators by hyperpolarizing VSM by activating K^+ channels. 20-HETE acts as a vasoconstrictor by reducing the open-state probability of Ca^{2+}-activated K^+ channels. Increased 20-HETE in VSM occurs after stimulation with angiotensin II (ANG II) and endothelin (McGiff and Quilley 2001). Decreased 20-HETE in VSM occurs after stimulation with nitric oxide (NO) and carbon monoxide (CO) (Roman *et al* 2000). Our goal is to develop sensitive and specific immunoassays for 20-HETE in biological samples, which should provide a simple non-invasive method for routine quantitative assessment of vasoconstriction mediated by 20-HETE.

2. Materials and methods

2.1 *Materials*

20-HETE was synthesized by the laboratory of John Falck, University of Texas Southwest Medical Center, USA. Evaluation of the purity of the relatively labile 20-HETE was done by thin-layer chromatography (TLC) after storage and prior to use in preparing ELISA standards and protein conjugates. 20-HETE (1 to 3 μg) was chromatographed using 20 × 20 cm, 250 μmol, silica gel plates (EM Science, Gibbstown, NJ, USA). The solvent system was 95% methylene chloride, 5% methanol followed by phosphomolybdic acid (PMA) staining. Based on this method, the 20-HETE starting material was > 95% pure.

2.2 *Preparation of four 20-HETE-protein conjugates*

The four conjugates used in this project 20-HETE-Tg 20-HETE-BSA 20-HETE-AP, and 20-HETE-HRP were prepared using the same cross-linking procedure (Bauminger and Wilchek 1980). Dried 20-HETE was dissolved in anhydrous dimethylformamide (DMF) (100 mM), and activated using N′N-dicyclohexylcarbodiimide (DCC) (25 mM) and N-hydroxysuccinimide (NHS) (50 mM) in DMF for 30 min at room temperature. Proteins Tg, BSA, AP, and HRP were dissolved in 0·1 M sodium bicarbonate, pH 9·6, 20 mg/ml. Activated 20-HETE was combined with each protein, typically 1 mg 20-HETE : 2 mg carrier protein, and allowed to react for 2 h at 4ºC. The conjugates were dialyzed exhaustively in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline (PBS), pH 7·4. Samples were aliquoted and stored under argon at – 80ºC. Protein concentrations were determined by bicinchoninic acid (BCA) protein assay.

2.3 *Preparation of anti-20-HETE*

Goat immune serum was prepared by Bethyl Laboratories (Montgomery, TX, USA). Two pre-bled (test bleed 0) goats were immunized with 0·150 mg of 20-HETE-thyroglobulin in a 1 : 1 emulsion of Freund's complete adjuvant : PBS. Seven booster immunizations, substituting Freund's incomplete adjuvant for the complete adjuvant, followed the primary immunization at 2, 4, 6, 8, 10, 15 and 17 weeks. Test bleeds 1–5 were performed at 5, 7, 9, 11, and 18 weeks, respectively, following the primary immunization.

2.4 *Determination of anti-20-HETE titer*

Anti-20-HETE dilutions were added to microtiter ELISA plates that were pre-coated with 20-HETE conjugated to BSA, 1 μg/well/0·2 ml 0·1 M carbonate–bicarbonate buffer, pH 9·6, 4ºC overnight. After incubation with the diluted test bleeds at 22ºC for 2 h, the plates were developed with donkey anti-goat IgG conjugated to HRP 22ºC for 1 h, followed by HRP substrate, 3,3′,5,5′-tetramethylbenzidine (TMB) for 20 min. The reaction was stopped with 3·0 N H_2SO_4, 50 μl/well, and the plates were read at 450 nm. Only one of two goats responded, with an end-point titer of 1/20,000 in the final bleed. Using a similar procedure and ELISA plates coated with unconjugated BSA, no significant anti-BSA activity was detected in any of the bleeds.

Antiserum was purified using a procedure that precipitated non-immunoglobulin protein with caprylic acid and subsequently precipitated immunoglobulin with saturated ammonium sulphate (Reik *et al* 1987).

3. Results

3.1 *Anti-20-HETE ELISA*

We obtained high titer goat anti-20-HETE in one of two goats by immunizing with 20-HETE-Tg and screening the pre-bleeds and test-bleeds with 20-HETE-BSA- or BSA only-coated ELISA microtiter wells. Anti-20-HETE-BSA titer increased with each test bleed and no significant anti-BSA activity was found (data not shown).

3.2 *20-HETE-AP competitive ELISA and 20-HETE-HRP competitive ELISA*

In a competitive ELISA assay, there is an inverse relationship between signal strength and free analyte concentration. Competitive ELISAs are represented frequently by the log × transformation of a 4-parameter logistic equation (4PL),

$$y = d + (a–d)/(1 + (x/c)^b),$$

wherein 'x' is the concentration of free analyte, 'y' is the signal, 'a' is the maximum signal asymptote, 'b' is the

slope-related term, '*c*' is the concentration of analyte at the inflection point, and '*d*' is the minimum signal asymptote. The signal can also be normalized by computing B/B_0, wherein the maximum signal is B_0 (no free analyte present) and signals obtained in the presence of un-conjugated hapten are expressed as B/B_0. Note that the *c* term of the 4PL is equivalent to 50% B/B_0 (Christopoulos and Diamandis 1996; Sasaki and Mitchell 2002). After coating purified anti-20-HETE onto Nunc Maxisorb microplates, the colorimetric ELISA was developed by adding free 20-HETE standards and either 20-HETE-AP (figure 1) or 20-HETE-HRP (figure 2). ELISAs were also incubated without (figures 1A, 2A) or with (figures 1B, 2B) a proprietary enhancer solution that reduced interferences due to nonspecific binding.

The 20-HETE colorimetric ELISAs exhibited 80% B/B_0 values of 0·5 and 0·7 ng/ml for AP ELISA without and

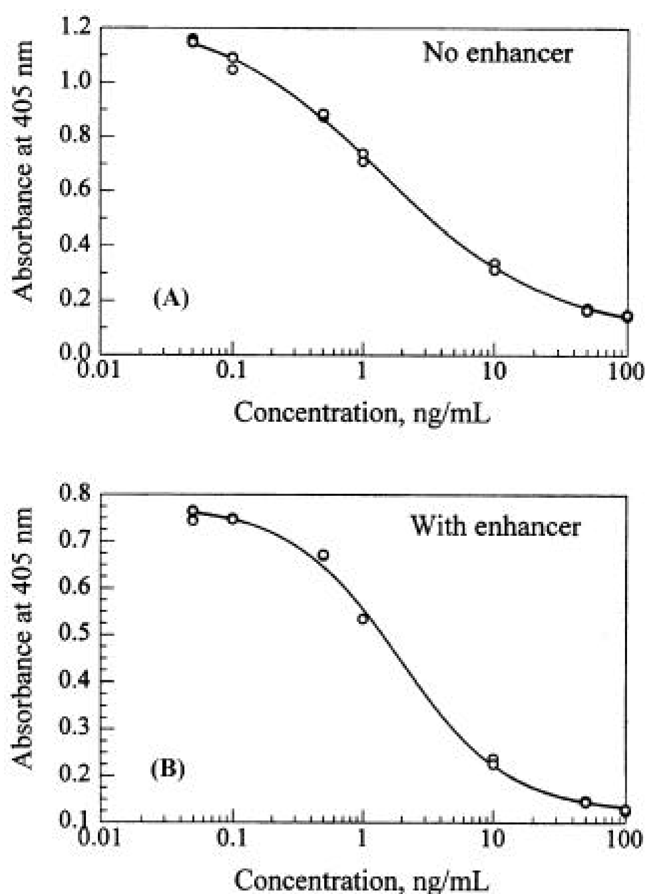

Figure 1. 20-HETE-AP competitive colorimetric ELISA standard curves. **(A)** Without enhancer: B/B_0, 1·5 ng/ml at 50%, 0·5 ng/ml at 80%; $r^2 = 0{\cdot}99$; signal range 0·100–1·200 at 0–100% B/B_0. **(B)** With enhancer: B/B_0, 2·0 ng/ml at 50%; 0·7 ng/ml at 80%, $r^2 = 0{\cdot}99$, signal range 0·100–0·800 at 0–100% B/B_0.

Briefly, the ELISA plate was precoated with goat anti-20-HETE IgG, 0·002 mg/well, 0·1 M carbonate/bicarbonate buffer, pH 9·6, 4ºC, overnight. The plate was blocked with 10% milk in phosphate buffered saline, 1 h, room temperature and washed with 0·1 M Tris buffered saline-0·01% Tween 20. Competing free 20-HETE standards, 0–100 ng/ml, and 20-HETE-AP 30 ng/well were added to the wells for 2 h, room temperature with and without enhancer. After washing the plate, AP substrate pNPP was added. The AP reaction was stopped after 60 min with 3 N NaOH and the plate read using a BioTek plate reader with a 405 nm filter. A 4-parameter logistic fit was used to analyse the data.

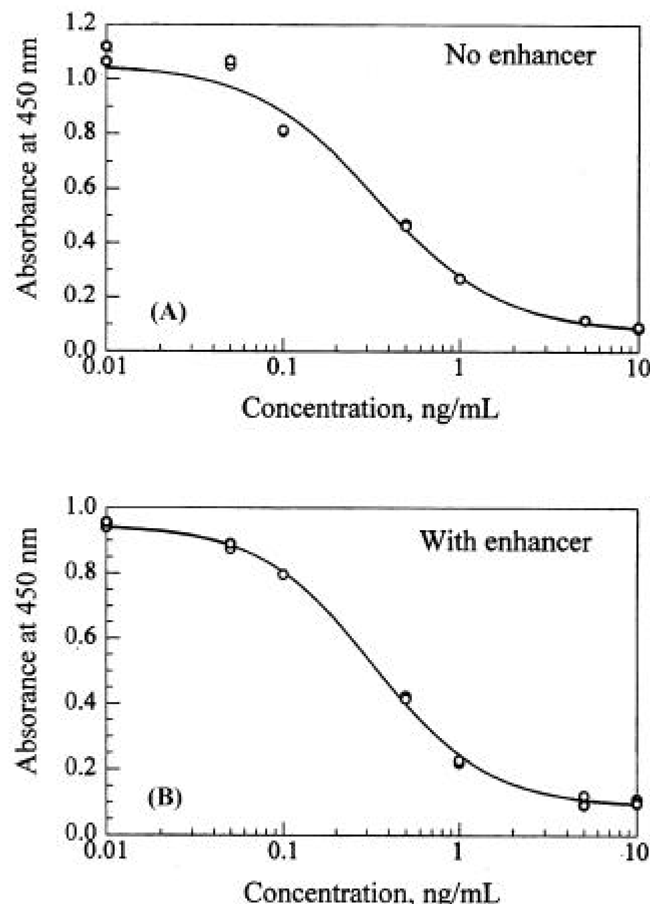

Figure 2. Competitive colorimetric 20-HETE ELISA standard curves. **(A)** Without enhancer: B/B_0, 0·5 ng/ml at 50%, 0·2 ng/ml at 80%; $r^2 = 0{\cdot}98$; signal range, 0·050–1·00 at 0–100% B/B_0. **(B)** With enhancer: B/B_0, 0·3 ng/ml at 50%, 0·1 ng/ml at 80%; $r^2 = 0{\cdot}99$, signal range, 0·090–0·900 at 0–100% B/B_0.

Briefly, the ELISA plate was pre-coated with goat anti-20-HETE IgG, 0·002 mg/well, 0·1 M carbonate/bicarbonate buffer, pH 9·6, 4ºC, overnight. The plate was blocked with 10% milk in phosphate buffered saline at 1 h, room temperature and washed with 0·1 M Tris buffered saline-0·01% Tween 20. Competing free 20-HETE standards, 0–10 ng/ml, and 20-HETE-HRP 400 ng/well were added to the wells for 2 h room temperature with or without enhancer. After washing the plate, HRP substrate TMB was added. The HRP reaction was stopped after 30 min with 3 N H_2SO_4 and the plate read using a BioTek plate read with a 450 nm filter. A 4-parameter logistic fit was used to analyse the curve data.

Table 1. Cross-reactivity of 20-HETE-AP-ELISA.

Lipid	Crossreactivity (%)
20-Hydroxyeicosatetraenoic acid (20-HETE)	100·00
Arachidonic acid (AA)	0·32
5(±)-Hydroxyeicosa-6E,8Z,11Z,14Z-tetraenoic acid (5-HETE)	0·07
8(±)-Hydroxy-5,9,11,14-eicosatetraenoic acid (8-HETE)	0·02
9(±)-Hydroxy-5,7,11,14-eicosatetraenoic acid (9-HETE)	< 0·02
11(±)-Hyrdroxy-5,8,12,14-eicosatetraenoic acid (11-HETE)	< 0·02
15(S)-Hydroxyeicosa-5Z,8Z,11Z,13E-tetraenoic acid (15-HETE)	< 0·02
(±)5,6-Epoxyeicosa-8Z,11Z,14Z-trienoic acid (5,6-EET)	0·02
(±)8,9-Epoxyeicosa-5Z,11Z,14Z-trienoic acid (8,9-EET)	0·04
(±)11,12-Epoxyeicosa-5Z,8Z,14Z-trienoic acid (11,12-EET)	< 0·02
(±)14,15-Epoxyeicosa-5Z,8Z,11Z-trienoic acid (14,15-EET)	0·02
(±)5,6-Dihydroxy-8Z,11Z,14Z-eicosatrienoic acid (5,6-DHET)	< 0·02
(±)8,9-Dihydroxy-5Z,11Z,14Z-eicosatrienoic acid (8,9-DHET)	< 0·02
(±)11,12-Dihydroxy-5Z,8Z,14Z-eicosatrienoic acid (11,12-DHET)	< 0·02
(±)14,15-Dihydroxy-5Z,8Z,11Z-eicosatrienoic acid (14,15-DHET)	0·02
Prostaglandin E_2 (PGE_2)	< 0·02
Prostaglandin F_{1a} (PGF_{1a})	< 0·02
8-epi-Prostaglandin F_{2a} (8-epi-PGF_{2a})	< 0·02

with enhancer, respectively; and 0·2 and 0·1 ng/ml for ELISAs employing HRP-conjugated 20-HETE without and with enhancer, respectively. The HRP-ELISA is somewhat more sensitive than the AP-ELISA. Addition of the enhancer has little effect on the sensitivity of either ELISAs. Of the 17 lipids tested for cross reactivity, thus far, there were no significant cross-reactions (table 1).

3.3 *Measurement of 20-HETE competitive in serum and urine*

A goat serum specimen was extracted using a C_{18} Sep-Pak® column (Waters® Corporation) described for PGE_2 extraction (Oxford Biomedical Research product EA02). The measured concentration of 20-HETE measured in the absence of enhancer was 150 ± 20 nM. The concentration of 20-HETE in normal unextracted human urine was 1·0 ± 0·2 nM. Analyses of 20-HETE in urine ± glucuronidase treatment are in progress.

4. Discussion

The assay sensitivity range of the colorimetric 20-HETE ELISA (0·3–1·4 nM) allows one to measure serum concentrations of 20-HETE in experiments (i) using normal microvasculature with values of approximately 100 nM (Gebremedhin *et al* 2000), and (ii) using 20-HETE supplementation (10 nM) designed to significantly reduce the diameter of rat renal afferent arterioles (Imig *et al* 1996).

ELISA measurements of urinary 20-HETE in the presence of enhancer are likely detecting greater than free urinary 20-HETE, since free urinary 20-HETE, is reported to be ≤ 0·2 nM by GC/MS (Prakash *et al* 1992). The other immunoreactive compounds are probably either untested cross-reacting lipid species (table 1) or the 20-HETE glucuronide that represents 90% of urinary 20-HETE with 10% free 20-HETE. Experiments with glucuronidase-treated urine samples are in progress. The colorimetric competitive ELISA described offers an attractive simple, rapid and accurate alternative to GC/MS for quantitative measurements of 20-HETE.

Acknowledgements

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