Fluorescent HPLC assay for 20-HETE and other P-450 metabolites of arachidonic acid

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Maier, Kristopher G., Lisa Henderson, Jayashree Narayanan, Magdalena Alonso-Galicia, John R. Falck, and Richard J. Roman. Fluorescent HPLC assay for 20-HETE and other P-450 metabolites of arachidonic acid. Am J Physiol Heart Circ Physiol 279: H863–H871, 2000.—This study describes a fluorescent HPLC assay for measuring 20-hydroxyeicosatetraenoic acid (20-HETE) and other cytochrome P-450 metabolites of arachidonic acid in urine, tissue, and interstitial fluid. An internal standard, 20-hydroxyeicosapentaenoic acid, was added to samples, and the lipids were extracted and labeled with 2-(2,3-naphthalimido)ethyl trifluoromethanesulfonate. P-450 metabolites were separated on a C18 reverse-phase HPLC column. Coelution and gas chromatography-mass spectrometry studies confirmed the identity of the 20-HETE peak. The 20-HETE peak can be separated from those of dihydroxyeicosatrienoic acids, other HETEs, and epoxyeicosatrienoic acids. Known amounts of 20-HETE were used to generate a standard curve (range 1–10 ng, r² = 0.98). Recovery of 20-HETE from urine averaged 95%, and the intra-assay variation was <5%. Levels of 20-HETE were measured in 100 μl of urine and renal interstitial fluid or 0.1 mg of renal tissue. The assay was evaluated by studying the effects of 1-aminobenzotriazole (ABT) on the excretion of 20-HETE in rats. ABT reduced excretion of 20-HETE by >65% and inhibited the formation of 20-HETE by renal microsomes. The availability of this assay should facilitate work in this field.

20-hydroxyeicosatetraenoic acid; cytochrome P-450; high-performance liquid chromatography; fluorescent; epoxyeicosatrienoic acids; eicosanoids; kidney

Recent studies have indicated that arachidonic acid (AA) is primarily metabolized in the brain, kidney, lung, and vasculature by cytochrome P-450 enzymes to epoxyeicosatrienoic acids (EETs), dihydroxyeicosatrienoic acids (dihETEs), and 19- and 20-hydroxyeicosatetraenoic acids (19- and 20-HETE) (13, 21). 20-HETE and EETs are biologically active and have been implicated as paracrine factors and/or second messengers in the regulation of vascular tone, sodium and water excretion in the kidney, and airway resistance (13, 21). Despite the importance of P-450 metabolites of AA in the control of vascular tone and renal function, very little is known about the regulation of the concentrations of these mediators in tissue and biological fluids. Part of the problem has been the lack of a sensitive, inexpensive, and high-throughput assay to measure the endogenous concentration of these compounds. To date, gas chromatography-mass spectroscopy (GC-MS) with selective ion monitoring has been the only method available to measure the concentration of P-450 metabolites of AA in biological samples (5, 7, 20, 23, 25). GC-MS has been successfully used to measure 20-HETE and EETs in the urine of humans and rats, and the reported concentration of these mediators is in the range of 0.5–5 ng/ml. The urinary excretion of EETs has been reported to increase in rats fed a high-salt diet (5, 19) and in patients with toxemia of pregnancy (7). Moreover, the urinary excretion of 20-HETE is elevated in patients with hepatorenal syndrome (22) and in DOCA-salt hypertensive rats (18).

Although GC-MS is a reliable method for the measurement of P-450 metabolites of AA, the high cost for the purchase and maintenance of the instrumentation and difficulties in preparing the samples for analysis have limited the use of this technique. Indeed, the preparation of urine samples for GC-MS involves an organic extraction of the lipid fraction, separation of the EETs or HETEs fractions by thin-layer chromatography and reverse-phase HPLC, derivatization of the samples to the methyl or pentabenzylfluoro esters, and conversion of these esters to trimethylsilyl derivatives. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
(25). It also requires the synthesis and addition of a deuterated internal standard to the samples to correct for variable extraction and derivatization efficiencies. The extensive sample preparation reduces sample recoveries and the detection limits of this technique to the nanogram range (19, 23, 25).

In the last few years, many fluorescent HPLC-based methods have been described for the analysis of fatty acids following derivatization of the carboxyl or hydroxyl groups with anthryldiazomethane (ADAM), pyrenylidiazomethane (PDAM) (4), bromomethyl- and diethylaminocoumarin (2), 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate (15, 26, 27), and other dyes. Some of these studies have reported detection limits <10 pg for prostaglandins and fatty acids. The present study describes an adaptation of one of these labeling techniques, which now allows for the simultaneous measurement of 20-HETE and other cytochrome P-450 metabolites of AA in urine, tissue, and biological fluids.

The assay is relatively simple and inexpensive to perform, and the practical detection limit for 20-HETE, other HETEs, diHETEs, and EETs in biological samples is ~0.5 ng. With the use of the maximum gain of the fluorescence detector, the detection limit for 20-HETE is 1 pg.

METHODS

Reagents. All chemicals used were of analytic or HPLC grade. The fluorescent probes, 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate, 9-ADAM, 7-diethylaminocoumarin, 1-ADAM, 4-bromomethyl-6,7-dimethoxycoumarin, and 3-PDAM were all purchased from Molecular Probes (Eugene, OR). The catalyst N,N-diisopropylethylamine and 1-aminobenzotriazole (ABT) were purchased from Sigma Chemical (St. Louis, MO). 5,6-diHETE, 5,6-EET, and the 15-, 12-, and 5-HETE standards were from Biomol (Plymouth Meeting, PA). An internal standard, 19-hydroxynonadeca-5(Z),8(Z),11(Z),14(Z)-tetraenoic acid, and 8,9-, 11,12-, and 14,15-diHETE, 16-, 18-, 19- and 20-HETE, and 8,9-, 11,12- and 14,15-EET were synthesized by J. R. Falck (Univ. of Texas Southwestern Medical Center, Dallas, TX). Another internal standard, 20–5(Z),14(Z)-hydroxyeicosadienoic acid (WIT-002) was synthesized and kindly provided by Taisho Pharmaceutical (Saitama, Japan). HPLC-grade methanol was purchased from VWR (South Plainfield, NJ), and acetic acid was purchased from Fisher Scientific (Pittsburgh, PA).

Lipid extraction. An internal standard (100 ng/ml of WIT-002 (1), 19-hydroxynonadeca-5(Z),8(Z),11(Z),14(Z)-tetraenoic acid (1), or a nonbiologically relevant hydroxy fatty acid such as 10- or 16-hydroxyhexadecanoic acid was added to the samples. The acidic lipids were extracted with 3 vols of ethyl acetate after 0.25 ml of urine, 100 µg of renal tissue homogenate, or 100 µl of buffer collected from a microdialysis probe was acidified to pH 3.0 with formic acid. The samples were dried down under argon, reconstituted in 0.5 ml of 20% acetonitrile:water (pH 3.0), and applied to a Sep-Pak Vac 1 cc (catalog no. WA504955; Waters, Milford, MA) that was prewashed with 1 ml of water followed by 1 ml of acetonitrile and 1 ml of water. The column was washed twice with 1 ml of 30% acetonitrile:water to remove polar lipids and then eluted with 400 µl of 90% acetonitrile:water. The eluent was diluted with 900 µl of water and applied to a prewashed Sep-Pak Vac column and then eluted with 500 µl of ethyl acetate to capture the free fatty acids. The sample was taken to dryness under argon.

Fluorescent labeling and HPLC analysis of lipids. In preliminary experiments, standards and samples were derivatized with 9-ADAM (16), 1-ADAM (14), PDAM (4), 7-diethylaminocoumarin (3), and 4-bromomethyl-6,7-dimethoxycoumarin (2) using previously published techniques. Although we were able to completely derivatize the samples, we could not find a solvent system that would resolve labeled 20-HETE from other HETEs (15-, 12-, 5-) and 5,6-diHETEs using normal phase, C18, or C8 reverse-phase HPLC. The only label that we found that was capable of resolving these closely related structures was 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate. We initially attempted to label 20-HETE and other P-450 metabolites according to the method of Yasaka et al. (27) with 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate using potassium fluoride and 18-crown-6 or tetraethylammonium carbonate as a catalyst.

Although this technique works well with standards dissolved in organic solvents, the labeling of biologic samples is very inconsistent because the reaction is inhibited by moisture. We searched for another catalyst and found that N,N-diisopropylethylamine catalyzes this reaction much more consistently.

To label the fatty acids, we resuspended samples, which were extracted and dried under argon, in 20 µl of acetonitrile containing 36.4 mM 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate. N,N-diisopropylethylamine (10 µl) was added to catalyze the reaction. The sample was reacted for 30 min at room temperature. The reactions were dried down under argon, resuspended in 1 ml of 20% acetonitrile:water, and applied to a Sep-Pak Vac column. The column was washed with 6 µl of 50% acetonitrile:water solution to remove unreacted dye and eluted with 500 µl of ethyl acetate. The eluent was dried under argon, and the samples were resuspended in 100 µl of the HPLC mobile phase (methanol:water:acetic acid, 82:18:0.1 vol/vol/vol). A 20 µl aliquot of the derivatized sample was separated on a 4.6 × 250-mm Symmetry C18 reverse-phase HPLC column (Waters, Milford, MA) isocratically, at a rate of 1.3 ml/min using methanol:water:acetic acid at 82:18:0.1 vol/vol/vol as a mobile phase. We tested various other C18 reverse-phase HPLC columns and found that the Symmetry column was the only one that could resolve all of the labeled P-450 metabolites of AA from each other. Fluorescence intensity was continuously monitored using a fluorescence detector (model L-7480; Hitachi, Naperville, IL) at a medium gain sensitivity. The amount of 20-HETE in the sample was determined by comparing the area of the 20-HETE peak to that of an internal standard. Between samples, the column was flushed for 10 min with a solution containing methanol plus 50% tetrahydrofuran to remove any residual labeled fatty acids.

GC-MS confirmation of labeled 20-HETE peak. 20-HETE (10 ng) was added to a 1-ml sample and derivatized with 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate. The fraction containing the fluorescent peak (retention time 41 min) was collected and dried under argon. The derivatized sample was redissolved in 49.95% methanol, 49.95% water, and 0.1% formic acid and introduced into an electrospray mass spectroscopy source at a rate of 5 µl/min using a Harvard syringe pump (Harvard Apparatus, South Natick, MA). Mass spectral data were acquired over a mass-to-charge range of 200–1,200 at the rate of 5 s/scan with a Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, UK) fitted with an electrospray source.

Biological validation of the 20-HETE assay. Experiments were performed on 10- to 12-wk-old male Sprague-Dawley
rats and spontaneously hypertensive rats (SHR) purchased from Harlan Sprague Dawley (Indianapolis, IN). The rats were housed in pairs in a dedicated animal facility with a 12:12-h light-dark cycle and allowed free access to a standard rat chow and drinking water. All procedures were approved by the Animal Care and Use Committee at the Medical College of Wisconsin.

Effects of an irreversible P-450 inhibitor on the urinary excretion of 20-HETE. SHR and normotensive Sprague-Dawley rats were placed in special metabolic cages (model 650–00350; Nalgene, Rochester, NY) that efficiently separated urine from food and feces and were allowed to equilibrate for 5 days. After 5 days, the food was withdrawn from the rat to prevent contamination of the urine sample and a 24-h control urine sample was collected on dry ice. After the control sample was collected, food was returned to the rats, and after a 2-day recovery period, the rats received an intraperitoneal injection of ABT (50 mg/kg) followed by a second (50 mg/kg) injection 12 h later. Two hours after administration of the first dose of ABT, food was withdrawn and a 24-h experimental urine sample was collected on dry ice. After the urine was collected, the rats were killed with pentobarbital sodium (100 mg/kg ip), and the kidneys were collected. The renal cortex was separated from the renal medulla, frozen in liquid nitrogen, and stored overnight for measurement of the renal metabolism of AA. Briefly, the renal cortex was homogenized in a 10 mM potassium phosphate buffer (pH 7.7) containing 250 mM sucrose, 1 mM EDTA, and 10 mM magnesium chloride, and microsomes were prepared by differential centrifugation. P-450A enzyme activity was assayed by incubating renal cortical microsomes (0.5 mg) for 15 min at 37°C with [1-14C]AA (0.1 μCi, 42 μM; Amersham, Arlington Heights, IL) in 1 ml of a 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM NADPH as previously described (12). The reactions were terminated by acidification to pH 4 using 0.1 M formic acid and extracted with ethyl acetate. Metabolites were separated using a 25-cm × 2-mm inner diameter (Supelco, Bellefonte, PA) C18 reverse-phase HPLC column and a linear elution gradient ranging from acetonitrile:water:acetic acid (50:50:0.1 vol/vol/vol) to acetonitrile:acetic acid (100:0.1 vol/vol) over a 40-min period. The radioactive products were monitored using a radioactive flow detector (model 120; Radiomatic Instrument, Tampa, FL).

Measurement of 20-HETE levels in renal interstitial fluid of anesthetized rats. Male Sprague-Dawley rats were anesthetized with ketamine (30 mg/kg im) and Inactin (50 mg/kg ip). The femoral artery and vein were cannulated for measurement of blood pressure and intravenous infusions, and the ureters were cannulated for collection of urine. The animal received an intravenous infusion of 0.9% NaCl containing 1% albumin at a rate of 6 ml/h during the experiment. A microdialysis probe (Bioanalytical Systems, West Lafayette, IN) was implanted 3 mm deep into the renal cortex of the left kidney and perfused with sterile saline at a rate of 10 μl/min. The animal was allowed to equilibrate for 1 h, and urine and microdialysis fluid were collected on ice for 1 h.

Statistics. Data presented are means ± SE; n is the number of samples measured. The significance of differences in mean values was analyzed using a paired or unpaired t-test. A P < 0.05 was considered to be statistically significant.

RESULTS

Assessment of the efficiency of the extraction procedure and labeling reaction. Experiments were performed to determine the recovery of 14C-labeled 20-HETE following extraction from urine with ethyl acetate and partial purification using a Sep-Pak Vac column. Mean recovery of labeled 20-HETE averaged 95 ± 3% (n = 6).

The effects of dye concentration and reaction time on the efficiency of the fluorescent derivatization reaction were also evaluated. 14C-labeled 20-HETE was derivatized with 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate, and the fraction of fluorescently labeled and unreacted 20-HETE was determined by HPLC using a fluorescence detector (model L-7480, Hitachi) and a radioactivity detector (model 120; Radiomatic Instrument, Tampa, FL) arranged in series. The fluorescent and radioactive peaks coeluted (Fig. 1), indicating that the fluorescent peak was derivatized 14C-labeled 20-HETE. The absence of a radioactive peak at 10 min, which corresponds to the retention time of unreacted 14C-labeled 20-HETE, indicates that the fluorescent labeling reaction was complete under the present experimental conditions. In other experiments, we determined that the labeling reaction did not reach completion, and two radiolabeled peaks were seen when the concentration of dye in the reaction was reduced or the reaction time was shortened to 10 min.

Separation of fluorescently labeled 20-HETE. A typical HPLC chromatogram illustrating the separation of the 20-HETE peak from other structurally similar P-450 metabolites of AA is presented in Fig. 2A. Fluorescently labeled 20-HETE elutes with a retention time of 41 min and is clearly separated from the peaks corresponding to labeled 14,15- and 8,9-diHETE, 15-, 12-, and 5-HETE, and 8,9-EET. A more complete listing of the retention times of all the other biologically relevant P-450 metabolites of AA and other potential interfering endogenous fatty acids that were tested is presented in Table 1. Even some of the compounds that are quite difficult to resolve from unlabeled 20-HETE, such as 19-, 18-, and 16-HETE and 5,6- and 8,9-diHETE, can be easily resolved from fluorescently labeled 20-HETE using this HPLC system.

![Fig. 1. Reverse-phase HPLC chromatogram demonstrating coelution of fluorescent and radioactive peaks following derivatization of 14C-labeled 20-hydroxyeicosatetraenoic acid (20-HETE; 10,000 counts/min (cpm)) with 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate.](http://ajpheart.physiology.org/)
A typical chromatogram of a derivatized sample of urine collected from a conscious rat is presented in Fig. 2B. This sample contains peaks for 11,12-EET and 8,9-diHETE and 20-HETE. It also contains peaks corresponding to the retention times of the subterminal HETEs (18-, 17-, 16-, 15-, 12-, and 5-HETE), and EETs. The peak seen at 80 min corresponds to the retention time of the internal standard, WIT-002. There is a small peak that appeared at 70 min when blank samples containing only dye and catalyst were injected. It therefore represents either a contaminant in the reagents or a fluorescent product formed from the dye and the catalyst. Unfortunately, this contaminant coelutes with 11,12-EET, an important \( P \)-450 metabolite of AA. However, the magnitude of this blank peak is usually small (<150 mV) and can be subtracted from sample chromatograms by the computer.

Other experiments were performed to confirm that the peak that elutes at 41 min was labeled 20-HETE. In these experiments, 20-HETE (100 ng/ml) was added to a blank sample, extracted, and derivatized, and the 41-min peak was collected and analyzed using liquid chromatography (LC)-GC-MS. The results of these experiments, presented in Fig. 3, confirm that this peak consists of a compound with mass-to-charge ratios of 544, 566, and 584, corresponding to the expected mass-to-charge ratios of fluorescently labeled 20-HETE, 20-HETE plus a sodium ion, and 20-HETE plus a potassium ion, respectively.
**20-HETE assay.** Standard curves were generated, in which samples containing various amounts of 20-HETE (5–200 ng/ml) and 100 ng/ml of an internal standard, either WIT-002 or 19-hydroxynonadeca-5(Z),8(Z),11(Z),14(Z)-tetraenoic acid, were extracted and fluorescently labeled. Aliquots of these samples containing 1–10 ng of 20-HETE and 5 ng of the internal standard were separated by reverse-phase HPLC. The ratio of the areas of the 20-HETE and internal standard peaks were plotted against the amount of 20-HETE in the aliquot and are presented in Fig. 4. The ratio of peak areas was highly correlated to the

![Image](https://via.placeholder.com/150)

Fig. 3. Liquid chromatography-gas chromatography chromatogram of the 41-min HPLC peak corresponding to the retention time of fluorescently labeled 20-HETE. The analysis was conducted in methanol:water:formic acid (49.95%:49.95%:0.1% vol/vol/vol) via electrospray using a Micromass Quattro II triple quadrupole mass spectrometer at a scan rate of 5 s/scan. The peak at 544 represents derivatized 20-HETE, the peak at 566 is the derivatized 20-HETE plus a sodium ion, and the peak at 582 is derivatized 20-HETE plus a potassium ion. m/z, Mass-to-charge ratio.

![Image](https://via.placeholder.com/150)

Fig. 4. Standard curves relating the ratio of areas of peaks to 20-HETE and internal standards, either 19-hydroxynonadeca-5(Z),8(Z),11(Z),14(Z)-tetraenoic acid (19-OH) or WIT-002. In these experiments various concentrations of 20-HETE between 1 and 10 ng/ml were added to samples containing 5 ng/ml of the internal standard. The samples were extracted and reacted with 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate, and fluorescent peaks were resolved by C18 reverse-phase HPLC using an isocratic elution with 82% methanol:water at a rate of 1.3 ml/h. Correlation coefficients averaged 0.99 for the curve relating 20-HETE and 19-OH (n = 3 samples) and 0.99 for the curve relating 20-HETE and WIT-002 (n = 3 samples).
expected amount of 20-HETE in the sample \( (r^2 > 0.98) \). The slopes of the curves were 0.19 for WIT-002 and 0.26 for 19-hydroxydocosahexaenoic acid. These coefficients are within errors associated with the addition of equal nanogram amounts of standards to samples and, for all practical purposes, indicate that 20-HETE and these two closely related analogs label with equal efficiency. This is not the case with all compounds. For example, we found that other fatty acids that do not have a double bond near the carboxyl group, such as linolenic and \( \gamma \)-linolenic acids, and other unsaturated fatty alcohols, such as 10-hydroxyeicosanoic acid, 16-hydroxyeicosanoic acid, or 15-hydroxyeicosanoic acid, label with a much higher efficiency (typically 3:1) than 20-HETE, HETEs, and EETs. They are also not extracted from biological samples with the same efficiency as 20-HETE.

Previous studies have indicated that the majority of 20-HETE and other HETEs in human urine is excreted as a glucuronide (20). We therefore examined the effects of treatment of rat urine samples with glucuronidase (50 units) at 37°C for 1 h on the concentration of 20-HETE. The results presented in Fig. 5 indicate that the concentrations of 20-HETE measured before and after treatment of samples of rat urine with glucuronidase were not significantly different.

We also measured 20-HETE levels after adding known amounts of this compound to urine samples and determined the intra-assay variation of the assay. Mean recovery of 20-HETE added to samples of rat urine was 95 ± 3% \( (n = 6) \). Intra-assay variation in repeated measurements of the same sample of urine made over a period of several days averaged 4.7 ± 1% \( (n = 6) \).

Effect of a P-450 inhibitor on the urinary excretion of 20-HETE. The effect of ABT on urinary excretion of 20-HETE in SHR and Sprague-Dawley rats is presented in Fig. 6. Control urinary excretion of 20-HETE was significantly higher in SHR \( (n = 5) \) than in Sprague-Dawley rats \( (n = 4) \) and averaged 1,334 ± 337 and 872 ± 338 ng/day, respectively. The excretion of 20-HETE fell by 65 ± 10% after administration of ABT to SHR and by 74 ± 9% in Sprague-Dawley rats. The change in the urinary excretion of 20-HETE paralleled the fall in the renal production of 20-HETE in microsomes prepared from the kidneys of these animals. In this regard, 20-HETE production by renal cortical microsomes averaged 459 ± 192 and 12 ± 4 pmol \( \cdot \) min \(^{-1} \) \( \cdot \) mg \(^{-1} \) in vehicle- and ABT-treated SHR \( (n = 5) \), respectively. Similarly, 20-HETE production fell from 324 ± 25 to 25 ± 3 pmol \( \cdot \) min \(^{-1} \) \( \cdot \) mg \(^{-1} \) in microsomes prepared from the kidneys of vehicle- and ABT-treated Sprague-Dawley rats \( (n = 4) \).

Measurement of 20-HETE levels in renal interstitial fluid and renal cortical tissue. Experiments were also performed to determine whether the assay was sensi-
tive enough to measure the levels of 20-HETE and other P-450 metabolites of AA in 100 µl of fluid collected from a microdialysis capsule acutely implanted in the renal cortex and brain of rats and perfused with sterile saline at 5 µl/min. 20-HETE, diHETEs, and EETs could be detected in samples collected from both the kidney and the brain. In the kidney, basal 20-HETE concentration averaged 3.1 ± 0.3 ng/ml (n = 4). This finding compared with a concentration of 20-HETE in renal cortical tissue of 1.8 ± 0.3 ng/g of tissue (n = 3). In microdialysis fluid collected from the brain of rats, we found that the concentrations of 20-HETE and diHETEs averaged 27 ± 3 and 39 ± 1 ng/ml, respectively (n=3).

**DISCUSSION**

The present study describes a simple, reliable, and inexpensive assay for the measurement of 20-HETE and the other major P-450 metabolites of AA present in urine, renal tissue, and both renal and brain interstitial fluid. The major advantage of this technique is that an internal standard that has a similar extraction and labeling efficiency as 20-HETE is added directly to the sample. Thus the amount of 20-HETE injected on the HPLC column can be directly determined by comparing the area ratio of the 20-HETE and internal standard peak without having to generate a standard curve or correct for differences in extraction efficiency, sample recovery, or labeling efficiency between samples. Utilizing this approach, we demonstrated that there was a highly significant linear correlation in a standard curve relating the amount of 20-HETE injected on the HPLC over a range from 1 to 10 ng and the area ratio of the 20-HETE and internal standard peak. The slopes of these lines indicate that the recovery and labeling efficiency of 20-HETE and the internal standards are essentially equal and yield a ratio of 1 when equal amounts (5 ng) of 20-HETE and internal standard are injected on the HPLC column. In other experiments, we found that the sensitivity of the assay can be greatly expanded by using the high gain setting of the fluorescence detector (100× instead of 10×). Under these conditions, the range of the standard curve could be reduced to 1–100 pg. However, this degree of sensitivity is not required for routine measurement of 20-HETE and other P-450 metabolites of AA in urine and renal tissue because the basal levels are quite high (10–100 ng/ml), and it only introduces more noise into the measurements.

In other experiments, we found that the reproducibility of the assay is excellent. The coefficient of variation of 20-HETE levels in repeated measurements on the same sample averaged only 4.7 ± 1% (n = 6). This high degree of reproducibility of the assay is due in large part to the unique catalyst that we used, which efficiently labels fatty acids even in the presence of moisture. Most of the previous work on labeling fatty acids with 2-(2,3-naphthalimido)ethyl trifluromethanesulfonate used potassium fluoride and 18-crown-6 as the catalyst (27). In the present study, we found that only standards that were dissolved in organic solvents and were completely dry after extraction would label using this catalyst. However, the labeling of biological samples was very inconsistent, because it is difficult, if not impossible, to completely remove moisture from biological samples containing high concentrations of salts, protein, and other hydroscopic compounds.

The assay was tested by measuring the recovery of 20-HETE levels after known amounts were added to samples of urine. We found that the recovery of the added 20-HETE to urine was complete and averaged 95 ± 3%. Further experiments were performed to confirm that the labeled 20-HETE peak can be resolved from all other known P-450 metabolites of AA, including 19-, 18-, 16-, 15-, 12-, and 5-HETE, 5,6-diHETE, and EETs. The results presented in Fig. 2 and Table 1 indicate that all of these compounds can be separated from 20-HETE after labeling, and most of them can be resolved from each other. This finding indicates that this technique can be used to simultaneously measure the concentration of other P-450 metabolites of AA in biological samples. The only exceptions are the subterminal HETEs (18-, 17-, and 16-HETE), which coelute at 45 min and cannot be resolved from each other. The only other problem is the small fluorescent peak that appears in blank samples at 70 min (averaging <150-mV deflection), which can interfere with the measurement of 11,12-EET. However, by running blank samples and using background subtraction, a computer data collection system can readily remove this interfering signal.

The utility of this assay to provide meaningful biological information in vivo was tested by measuring the effects of ABT on the urinary excretion of 20-HETE in SHR and Sprague-Dawley rats. This compound was chosen because Su et al. (24) have recently reported that a single 50 mg/kg intraperitoneal injection of ABT selectively inhibits the formation of 20-HETE in microsomes prepared from the kidneys of rats. In the present study, the basal excretion of 20-HETE was 53% higher in the SHR than in Sprague-Dawley rats. This finding is consistent with previous observations (10, 11, 17) that the renal production of 20-HETE is elevated in the kidney of SHR. ABT reduced the urinary excretion of 20-HETE by 65% in SHR and 74% in Sprague-Dawley rats, respectively. Similarly, the excretion of other P-450 metabolites of AA, such as diHETEs, the subterminal HETEs, and EETs, were also reduced by ABT, but the percent reduction in the excretion of these other compounds was much less than that seen with 20-HETE. This observation supports the view that ABT is more effective at inhibiting the P-4504A enzyme that produces 20-HETE in the kidney than it is at inhibiting other P-450 isoforms in the kidney or the formation of 20-HETE in the liver (24).

It is interesting that 20-HETE was still excreted in the urine of the ABT-treated rats even though the production of 20-HETE in microsomes prepared from the renal cortex of these animals was greatly reduced. This may be due to storage of P-450 metabolites of AA in the phospholipid pools in the kidney. Indeed, Carroll...
et al. (6) recently reported that 20-HETE and subterminal HETEs are stored in the kidney and that large quantities of these compounds are released in response to agonists that activate phospholipases, such as vasopressin, endothelin, and angiotensin II. Others have reported that EETs are incorporated and can be released from phospholipid pools in platelets, white blood cells, and vascular tissue (8, 9). Thus it is likely that it takes some time to completely deplete the pools of 20-HETE and other P-450 metabolites of AA in the kidney following acute inhibition of P-450 activity. It is also possible that ABT is less effective in inhibiting the formation of 20-HETE by blood elements and in other organs than the kidney (24). Therefore, some of the 20-HETE that appears in the urine after ABT treatment may be blood borne and filtered and/or secreted by renal tubules.

The concentration of 20-HETE in the urine of rats and the 24-h urinary excretion of 20-HETE reported in the present study are much higher than those reported by others using GC-MS techniques (5, 7, 19). Both techniques are nearly identical in principle, in that they rely on the inclusion of an internal standard for quantitation of the 20-HETE peak and utilize either GC or HPLC to resolve the 20-HETE peak from potential interfering compounds in the sample, such as 19-, 18-, 17-, 16-, and 15-HETE. In the present study, we demonstrated that labeled 20-HETE can be resolved from all these potential interfering compounds. Moreover, we found that ABT reduced the concentration of 20-HETE in the urine to very low levels and that the recovery of 20-HETE added to urine samples was complete. These results could not have been achieved if there had been a major interfering compound that would have elevated the apparent levels of 20-HETE determined using our fluorescent assay.

The possibility remains that the differences between the concentration of 20-HETE measured in the urine of rats in the present and previous studies simply reflect strain differences, diet differences, or environmental differences. For example, starvation induces P-450 4A enzymes, and we removed the food from our rats over-night. However, we feel the primary reason for the discordant results are differences in the handling of the animals and the collection of the urine samples before analysis. We have found that rats typically will not eat or drink normally for several days when placed in a metabolic cage. This reduces urine output and greatly increases the losses of 20-HETE in the cage. Indeed, we found that the urinary excretion of 20-HETE was fivefold lower when the urine samples were collected just after rats were placed in metabolic cages compared with the levels seen when the rats were given sufficient time to adapt to their new surroundings and urine output was much greater. The second issue is that we used Nalgene metabolic cages, which are far more efficient at preventing fecal and food contamination of urine samples than the traditional wire mesh metabolic cages. Food was also removed from the rats during the urine collection to prevent any contamination of the samples. This was done because the addition of powdered and crumbled rat chow to urine binds 20-HETE and other P-450 metabolites of AA and greatly diminishes the free concentration. Finally, the urine in the present study was collected into glass tubes in insulated boxes containing dry ice so that it was immediately frozen. This is necessary because there is tremendous breakdown and loss of 20-HETE (~90%) when it is added to rat urine and allowed to stand at room temperature overnight.

Perspectives

Recent studies have indicated that 20-HETE and other P-450 metabolites of AA play an important role in the regulation of renal function, vascular tone, and the long-term control of arterial pressure. However, very little is known about the factors that influence the concentrations of these substances in the kidney and other tissues because of the lack of simple and cost-effective assays for the measurement of these compounds in biological fluids. The present study describes a new fluorescent HPLC assay for 20-HETE that can be used to simultaneously measure the other major P-450 metabolites of AA in the sample as well. The availability of this assay opens new vistas for progress in understanding the physiological role of P-450 metabolites of AA.

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