Immunoaffinity Resin for Purification of Urinary Leukotriene E₄

Jay Y. Westcott,¹ Kirk M. Maxey, † Jim MacDonald, † and Sally E. Wenzel *

¹National Jewish Medical and Research Center, 1400 Jackson St, Denver, CO 80206, Division of Pulmonary and Critical Care Medicine, University of Colorado Health Sciences Center, Denver, CO 80206, USA; ¹Cayman Chemical Company, 690 KMS Place, Ann Arbor, MI 48108, USA

Urinary leukotriene E₄ (LTE₄) has been used as an index of total leukotriene synthesis. A wide variety of methods have been applied to measure LTE₄ which has made direct comparison of urinary levels reported by different laboratories difficult. A new peptidoleukotriene immunoaffinity resin was utilized for urinary LTE₄ purification in a method that is easy and inexpensive, utilizing commercially available reagents. This method is described and compared to other methods. LTE₄ (50–250 pg/mL) added to a urine extract was quantitatively recovered using the immunoaffinity resin. Similarly, LTE₄ (50–400 pg/mL) added to urine was recovered between 63 and 76%. The coefficient of variation of samples purified and quantified on the same or on different days ranged from 8–10%. There was a strong correlation (r² = 0.95) between LTE₄ concentrations determined after immunofiltration and immunoaffinity purification. Although there was a good correlation between urinary LTE₄ levels measured without purification compared to after immunoaffinity purification, the high y-intercept of 179 indicates the presence of interfering substances in unpurified urine. Urinary LTE₄ in normal healthy adults was 80 ± 7 pg/mg creatinine, similar to that previously reported following HPLC or immunofiltration purification. Urinary LTE₄ was also measured in healthy children (age 3–12) and found to be 103 ± 9.

Keywords: Immunoaffinity resin; urine; leukotriene E₄

¹Address correspondence to: Jay Y. Westcott, National Jewish Medical and Research Center, 1400 Jackson St, Denver, CO 80206; Phone: 303-398-1919; Fax: 303-398-1851; E-mail: Westcottj@njc.org
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Introduction

The peptidoleukotriene eicosanoids have been implicated in mediating symptoms of inflammatory diseases such as asthma [1–3]. Leukotriene E₄ (LTE₄) is a major leukotriene metabolite in urine. The concentration of this metabolite in urine can be utilized as an index of leukotriene synthesis after normalization to creatinine excretion [4–6]. This rationale has been shown to be appropriate as long as liver and kidney function are normal in the persons being studied. Increased LTE₄ synthesis as evidenced by increased urinary LTE₄ excretion has been reported in subgroups of asthmatics [7,8], rheumatoid arthritis patients [9], patients with coronary artery disease [10], and patients with lupus [11].

Because of the potential importance of leukotrienes in diseases, sensitive methods to accurately and easily measure LTE₄ in urine are necessary. The most widely utilized methods for LTE₄ analysis involve concentration of urine over an octadecylsilyl cartridge, HPLC purification, and quantitation by immunoassay [6,12]. This assay is labor intensive, requires expensive equipment and requires the use of a radiolabeled internal standard. Another method for analysis of urinary LTE₄ involves the direct quantification of urine by immunoassay [13,14]. Kumlin et al. reported that relative differences in urine LTE₄ levels could be obtained without any purification of urine. This methodology is sensitive, easy, and inexpensive, but accuracy is suspect due to the presence of other cross-reacting substances present in urine. Recently, an immunofiltration purification method was described that is easy and sensitive [15], but the reagents for this assay are not generally available.

The utilization of these diverse methods to purify and quantify urinary LTE₄ make it impossible to accurately compare measurements made in different laboratories [12]. With the current availability of leukotriene synthesis inhibitors and receptor antagonists it is important to be able to determine if a subject has normal or elevated leukotriene synthesis. To be able to do this, a universal method is required such that similar levels can be obtained regardless of where the assay is performed. This method should be relatively easy and inexpensive, and utilize reagents that are universally available. The recent commercial availability of a peptidoleukotriene immunoaffinity resin has allowed such a universal assay method. In this manuscript we describe the application of immunoaffinity purification to LTE₄ analysis and compare it with other commonly utilized methods. Normal levels for healthy adults and children were also measured and compared with previously determined levels.
Materials and Methods

Materials

The peptidoleukotriene immunoaffinity resin as well as most of the reagents utilized for enzyme immunoassays were from Cayman Chemical (Ann Arbor, MI). Tritiated LTE₄ was from New England Nuclear (Boston, MA). Immunoassay #2 utilized a mouse monoclonal peptidoleukotriene antibody purchased from PerSeptive Biosystems (Framington, MA) and goat anti-mouse IgG as a plate coating antibody (Zymed Laboratories, Inc, South San Francisco, CA). HPLC grade methanol was from Fisher, Scientific (Pittsburgh, PA). Other chemicals were of reagent grade or better.

Synthesis of the Immunoaffinity Resin

The antibody utilized in the immunoaffinity resin was a mouse monoclonal antibody produced in response to LTC₄ conjugated to keyhole limpet hemocyanin. Antibody was produced in the ascites fluid of BALB-C mice and purified by ammonium sulfate/caprylic acid precipitation. The purified antibody cross-reacted strongly with LTC₄ (90%), LTD₅ (39%), LTE₄ (39%), and N-acetyl LTE₄ (46%). The antibody did not cross react well with LTB₄ (7%) or 20-COOH LTE₄ (<1%). This purified antibody (50 mg) was dissolved to 10 mL of 0.1 M sodium carbonate buffer (pH 8.3) containing 0.5 M saline and added to 10 mL of the same buffer containing 3 g of CNBr-activated Sepharose 4B. The mixture was gently stirred for 1 h at room temperature, followed by the addition of 25 mL of 0.1 M Tris-HCl (pH 8.0). The resin was collected on a sintered glass filter and washed extensively with 0.1 M acetic buffer (pH 4.0). Unconjugated Sepharose was added to standardize the preparation at 375 μg IgG/mL of gel and the resin stored in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5 M saline and 0.05% sodium azide.

Utilization of Peptidoleukotriene Immunoaffinity Resin

In initial experiments, buffer or urine (1 mL) was spiked with unlabeled LTE₄ (0–100 ng/mL) and/or [³H]LTE₄ (specific activity 165 Ci/mMol, New England Nuclear, Boston, MA). Immunoaffinity resin was utilized as a 1/1 (v/v) mix of resin and resin storage buffer. Resin was added in concentrations ranging from 0 to 100 μL/mL of sample. Samples were vortexed and then mixed for an additional 2–60 min. Tritium remaining in the supernatant following centrifugation was measured by scintillation spectrophotometry. Similarly, the release of radioactivity was also determined after the resin was washed and after addition of methanol to the resin.

The main protocol for urine LTE₄ purification utilizing the immunoaffinity resin was as follows. Urine samples were frozen at −20°C within 16 h after collection. Interim storage was at 4°C although short periods at
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room temperature occurred. Storage of urine in this fashion does not result in substantial leukotriene degradation (15). On the day of assay, samples were thawed at room temperature or in warm water. Each sample was centrifuged at 6,000 rpm for 3–5 min. One milliliter of urine supernatant was removed from each sample and added to a 1.5 polypropylene centrifuge tube containing 20 μL of the leukotriene immunoaffinity resin (50% mix of resin suspended in buffer) unless stated otherwise. Tubes were gently mixed for 1 h at room temperature. Tubes were centrifuged at 10,000 rpm for 4 min. to separate the resin. The supernatants were carefully removed by pipetting and discarded. The resin from each sample was washed with 1 mL of phosphate-buffered saline (PBS) (0.9% saline in 100 mM potassium phosphate buffer, pH 7.4). To each resin pellet containing approximately 10–20 μL of residual wash buffer, 500 μL of cold methanol was added. The samples were vortexed and allowed to sit for between 5 min. and 16 h at −20°C. The methanol caused the dissociation of leukotrienes from the antibody/resin. Samples were centrifuged at 10,000 × g for 5 min. and the methanolic supernatants removed and pipetted into new tubes. To ensure that all resin had been removed, samples were routinely centrifuged a second time at 10,000 × g and the supernatants decanted off into clean tubes.

The tubes were inserted into a Speed-Vac concentrator and the methanol removed under vacuum and low heat. This typically required about 1 h. The drying time was not extended for long periods as this can cause leukotrienes to degrade. To each sample, 0.5 mL of assay buffer (0.5 M sodium chloride, 0.1 M potassium phosphate, 1.5 mM sodium azide, 1 mM EDTA, 0.1% bovine serum albumin (BSA), pH 7.4) was added and each sample vortexed. The actual quantitation was performed utilizing an immunoassay (immunoassay #1 unless stated otherwise) which was initiated immediately. In most cases, urinary LTE₄ concentrations were normalized to creatinine levels in urine. Creatinine was measured spectrophotometrically utilizing a commercially available kit (Sigma Chemical, St Louis, MO).

To study the ability of this purification method to recover LTE₄, a urine sample was spiked with 0–250 pg/mL of LTE₄. This urine sample was previously applied to a Sep-Pak cartridge and the fluid passing through the cartridge was utilized. This urine extract has the advantages of low background LTE₄ while maintaining other compounds which stabilize LTE₄ (15). The fluid eluting through the cartridge was either spiked with 250 pg/mL LTE₄ or nothing. The urine containing the LTE₄ was aliquoted into 3 tubes and further diluted with the extracted urine to either 200, 150, 100, or 50 pg LTE₄/mL. Each LTE₄ concentration was purified and quantified 3 separate times. Since the recovery of LTE₄ from urine could be influenced by the presence of these other cross-reacting substances, similar recovery experiments were performed in four urine samples (from asthmatic children aged 7–12 years) spiked with 0, 50, 100,
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200, or 400 pg/mL LTE$\textsubscript{4}$. These samples were purified with the immunoaffinity resin and quantified by enzyme immunoassay. For comparison, the urine was also diluted 1/4 with immunoassay buffer and LTE$\textsubscript{4}$ determined without purification.

The reproducibility of the assay was investigated in several ways. To study the intraassay variability, one urine sample (from an asthmatic child, creatinine = 0.87 mg/mL) was aliquoted into seven tubes and purified and quantified separately during one day. The quantification was performed on one assay plate. To determine the reproducibility of the assay on different days, one urine sample (healthy control, creatinine = 1.6 mg/mL) was spiked with 1 ng/mL LTE$\textsubscript{4}$ and aliquots stored at $-20^\circ$C. One aliquot was assayed on each of seven separate occasions. An additional reproducibility experiment involved performing serial dilutions of one sample and taking each diluted sample through both purification and immunoassay quantitation.

Comparison of Immunoaffinity LTE$\textsubscript{4}$ Purification with Other Methods

This new method for urinary LTE$\textsubscript{4}$ purification and quantitation was compared with two other methods of LTE$\textsubscript{4}$ determination. The first of these utilized an immunoassay to quantify LTE$\textsubscript{4}$ in urine without prior purification [13]. The second comparison was with an immunofiltration method of urinary LTE$\textsubscript{4}$ purification which was recently validated [15]. The urine samples utilized were a mixture of freshly collected urine and urine that had been stored for up to 60 days at $-20^\circ$C. The urine samples utilized for the main methodology comparison ($n = 17$) were obtained from healthy subjects, asthmatics treated in an emergency room, and an aspirin-intolerant asthmatic undergoing aspirin desensitization. One sample was spiked with 1000 pg/mL exogenous LTE$\textsubscript{4}$. This diversity of subjects and samples provided a wide range of urinary LTE$\textsubscript{4}$ concentrations.

The immunofiltration protocol has recently been described [15]. Briefly, urine samples (0.4 mL) were incubated with 50 µg (in 0.03 mL) of a mouse monoclonal antibody against peptidoleukotrienes. This antibody cross reacts with LTD$\textsubscript{4}$ (100%), LTC$\textsubscript{4}$ (96%), LTE$\textsubscript{4}$ (89%), and N-acetyl LTE$\textsubscript{4}$ (90%) [16]. After 4–5 h at room temperature, 0.4 mL of each sample was loaded onto a Centricon filter (10,000MW cut-off, Amicon Inc., Beverly, MA) placed in a 1.5 mL polypropylene tube supplied with the filter. Samples were centrifuged at 12,000 $\times$ g at 4 degrees for 30 min. The fluids passing through the filters were discarded. An additional 0.3 mL of PBS (0.1 M potassium phosphate in 150 mM sodium chloride, pH 7.4) was added to the top of each filter to wash what had been retained by the filter. Samples were centrifuged again for an additional 25 min. at 12,000 $\times$ g at 4°C. The filter holders were removed from each tube, an additional 35 µL of buffer added to each filter, and the filters inserted in an inverted manner into new tubes. The tubes were centrifuged at 1000 $\times$
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g for 3 min. The filters were removed and discarded. To the fluid remaining in the tubes, 0.5 mL of methanol was added and the samples stored at −20°C for 16–20 h. The samples were then centrifuged at 12,000 × g for 3 min. at room temperature. Supernatants were decanted into new 1.5 mL polypropylene centrifuge tubes and centrifuged for an additional 3 min. at 12,000 × g to ensure that all the precipitated protein was removed. The supernatants were decanted into another 1.5 mL polypropylene tube and put in a Savant Speed-Vac concentrator where the methanol was removed under negative pressure. The residue was resuspended in buffer and immunoassay initiated within 2 h.

Evaluation of the Enzyme Immunoassay for LTE₄ Quantitation

The immunoassay used for quantitation of LTE₄ (termed immunoassay #1 in comparison experiments) utilized reagents obtained from Cayman Chemical including plates coated with a mouse monoclonal anti-rabbit antibody, a specific rabbit polyclonal peptidoleukotriene antibody, an LTE₄- acetylcholinesterase tracer, and an LTE₄ standard. This antibody cross reacts 100% with LTC₄ and LTD₄, 67% with LTE₄ and 10.5% with N-acetyl LTE₄. The sensitivity of this assay was 10–20 pg/mL.

The basic enzyme immunoassay protocol was as follows. Samples (50 μL) or standards were added to wells in duplicate. Samples were generally assayed at two dilutions. These dilutions ranged from a 4/1 concentration of the initial urine to a 1/5 dilution. LTE₄ conjugated to acetylcholinesterase (50 μL) was added to each well as an enzyme tracer. Antibody (50 μL) was also added to wells and allowed to incubate for 5–18 h at room temperature. Wells were then washed five times with wash buffer and Ellman's reagent added. A stable yellow-colored product was produced that was proportional to the amount of enzyme present. The absorbance (405 nm) was read after 4–20 h and the absorbance of the samples were compared with that of standards by a computer program using a 4-parameter logistic fit for the calculation of sample LTE₄. These assays are flexible with respect to length of incubation and development and the choice can largely depend on the convenience of the person performing the assay.

We wanted to determine the importance of the main components of the immunoassay. The anti-leukotriene antibody was varied in a second enzyme immunoassay (immunoassay #2). This second immunoassay has been utilized in the authors laboratory for several years and has been previously described (6,15). This immunoassay is very similar to immunoassay #1 but utilizes a mouse monoclonal peptidoleukotriene antibody purchased from PerSeptive BioResearch Products [Cambridge, MA]. This antibody was utilized at a 1/10 dilution of that recommended for radioimmunoassay and has significant cross-reactivity to LTC₄ (55%), LTD₄ (100%), LTE₄ (51%), and N-acetyl LTE₄. Immunoassay plates [Immulon 4, Dynatech] were coated with a goat anti-mouse antibody [Zymed, CA]
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at 4 ug/mL. Sensitivity of the assay ranged from 35–70 pg/mL. The 17 urine samples utilized to compare purification methods were quantified with both immunoassays. The other main component of enzyme immunoassays, the enzyme-tracer conjugate was evaluated in another study. The LTE₄-acetylcholinesterase tracer was compared with a LTC₄-acetylcholinesterase tracer in samples purified by immunoaffinity resin.

Urinary LTE₄ Levels in Healthy Adults and Children

Spot urine samples were obtained from local healthy volunteers. Adults \( n = 14 \) were non-smokers ranging in age from 25–60. Children \( n = 20 \) ranged in age from 3 to 12 years.

Statistical Analysis

Recovery and method comparison experiments were compared by calculating the Pearson correlation coefficient. Linearity of fit was determined utilizing linear regression analysis. Reproducibility of the assay was determined by calculating the percentage standard deviation (coefficient of variation %). To determine if the LTE₄ levels were different in healthy adults and children, a Wilcoxon/Kruskal-Wallis Test was performed. Statistical significance was assumed when \( p < 0.05 \).

Results

Utilization of Peptidoleukotriene Immunoaffinity Resin

Initial experiments were performed to determine if the immunoaffinity resin could bind LTE₄ and how much could be bound. Tritiated LTE₄ (25,000 dpm/mL, 30 pg LTE₄/mL) was added to immunoassay buffer. Aliquots of 1 mL were pipetted into 1.5 mL polypropylene tubes and additional LTE₄ added (0 ng, 1 ng, 10 ng, or 100 ng). Each LTE₄ concentration was tested in duplicate. Immunoaffinity resin (100 ul) was added to each sample and the samples mixed for 2 h. At this time the resin was removed by centrifugation and the amount of radioactivity remaining in the supernatant determined. All the sample supernatants contained only 8–11% of the added tritium, indicating that the resin was capable of binding over 100 ng LTE₄/0.1 mL of immunoaffinity resin. A control sample containing no resin had a final level of radioactivity similar to that of the initial amount added so the radiolabeled leukotriene was being specifically bound to the resin. For additional proof that added LTE₄ was being bound by the resin, a portion of the supernatant from a sample to which 100 ng/mL of LTE₄ had been added was quantified by immunoassay and found to contain only 2.5 ng/mL (2.5% of the added LTE₄). The slight discrepancy between the percentage of added tritium and immunoactive LTE₄ in the supernatant is most likely due to impurities present in the starting \(^{3}H\)LTE₄.

Since this method will be primarily utilized to purify urinary LTE₄,
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Experiments were performed to determine the effect of the amount of resin added and time of mixing on the removal of LTE₄ from urine. Urine was spiked with 10,000 dpm/mL of [³H]LTE₄ and 2 ng/mL of unlabeled LTE₄. This concentration of LTE₄ was chosen because it approaches the highest level normally present in urine. Immunoaffinity resin (0–50 uL) was added to polypropylene centrifuge tubes (1.5 mL) with each condition examined in duplicate. Urine (1 mL) was added to each tube and the samples vortexed. The samples were continued to be mixed by slow rotation. After 2 min., samples were quickly centrifuged (3000 × g for 2 min.) and 0.1 mL of supernatant removed and counted for tritium. Samples were vortexed again and the mixing continued until 30 min. had passed since the addition of urine to the resin. Samples were again centrifuged and 0.1 mL removed for counting radioactivity. Mixing was then continued until 60 min. had elapsed. Samples were centrifuged again and another 0.1 mL counted for tritium. There was no change in the tritium in the supernatant of the two samples containing no immunoaffinity resin and this was chosen to equal 100% for comparison purposes. Figure 1 shows that the radiolabeled LTE₄ was removed from the urine by binding to the resin and that this process was dependent on the time of mixing and the amount of resin present.

To determine if the bound [³H]LTE₄ could be removed from the resin, the supernatants of the resin in these samples were removed and the resin pellets washed with 1 mL of PBS. This wash was removed after pelleting the resin by centrifugation and the supernatants counted. The wash contained only small amounts of radioactivity, totaling 1–5% of the initially added radioactivity. Methanol (0.5 mL) was added to each sample and mixed with the resin. The resin was removed by centrifugation and the methanolic supernatants removed and tritium determined. An excellent recovery of added radiolabel was found in the methanol supernatants that was inversely proportional to the amount of resin present. With 50 uL of added resin 8800 dpm were recovered in the methanolic supernatant while 8500 dpm were recovered in the samples to which 25 uL of resin was added and 7300 dpm in samples with 10 uL of resin added.

The experiments presented have demonstrated that this immunoaffinity resin is capable of binding LTE₄. To demonstrate that this resin could also be utilized to quantify LTE₄, a recovery experiment was performed in which a urine extract was spiked with 0–250 pg/mL LTE₄. This is a range of LTE₄ that is commonly found in urine. Each concentration of LTE₄ was tested in three separate samples. Samples were purified using 20 uL of the immunoaffinity resin and quantified using immunoassay #1. Figure 2 shows that the added LTE₄ was quantitatively recovered from these samples. The graph of added LTE₄ and recovered LTE₄ shows a high correlation (r² = 0.98) a y-intercept of 4 and a slope of 0.95.

A separate recovery experiment was performed in unextracted urine samples. Four previously frozen urine samples were spiked with 0, 50,
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**FIGURE 1.** The effect of amount of leukotriene immunoaffinity resin added and time of mixing on the binding of \[^{3}H\] \( \text{LTE}_4 \) to the resin. Urine spiked with 10,000 dpm \[^{3}H\] \( \text{LTE}_4 \) and 2 ng/mL \( \text{LTE}_4 \) was aliquoted into eight 1 mL samples. Immunoaffinity resin (10, 25, or 50 \( \mu \)L) or nothing (control) was added to duplicate tubes. Samples were vortexed and aliquots of supernatant following pellet centrifugation were collected after 2, 30, and 60 min. Samples were vortexed again and mixed between collection times. The tritium in the supernatants was determined and compared to control samples (100%).

100, 200, or 400 pg/mL of \( \text{LTE}_4 \). Urine creatinine was measured and ranged from 0.85 to 1.0 mg/mL in these samples. \( \text{LTE}_4 \) was measured in these samples following purification with the immunoaffinity resin [20 \( \mu \)L of 50% resin added to 1 mL samples]. \( \text{LTE}_4 \) was also determined in unpurified samples without purification. The results are shown in Figure 3. This figure shows that \( \text{LTE}_4 \) is lower following purification, most likely due to the presence of interfering cross reacting substances in unpurified urine. The recovery of added \( \text{LTE}_4 \) ranged from 63 ± 15% when 50 pg/mL of \( \text{LTE}_4 \) was added to 75 ± 6% when 100–400 pg/mL of \( \text{LTE}_4 \) was added. This is similar to recoveries by other claimed by other techniques (6,17–19).

Several experiments were performed to assess the reproducibility of the assay. One urine sample was divided into seven aliquots and purified separately on one day. The samples were then quantified in one assay. The values of \( \text{LTE}_4 \) for this sample (creatinine of 0.87) ranged from 133 to 167 pg/mL (mean = 149 ± 4.4 SEM). The coefficient of variation was 8%.

In another set of experiments, one urine sample was spiked with 1 ng/mL.
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Exogenous LTE₄ and aliquots stored at -20°C. This sample was purified and quantified on 7 separate occasions. The sample LTE₄ ranged from 952 to 1259 pg LTE₄/mL (mean = 1075 ± 37 SEM) with a coefficient of variation of 9%. In a third experiment, a sample with high creatinine (2.01 mg/ml) and elevated LTE₄ was purified without dilution and following a 1/2, 1/4, 1/8, and 1/16 dilution made with water. The level of LTE₄ measured in these samples was directly related to the dilution of the sample made prior to purification (r² = 0.98, Fig. 4).

Comparison of Immunoaffinity LTE₄ Purification with Other Methods

Urine samples (n = 17) were purified by binding to the immunoaffinity resin (40 uL resin) as well as by immunofiltration. The relationship between direct assay of unpurified urine and levels measured after immunoaffinity resin purification was examined first. Figure 5 shows this comparison. Although there was a good correlation between the two measurements (r² = 0.67), the high y-intercept (179) and slope of 1.47 indicates the presence of other cross reacting substances present in unpurified urine.

The comparison of the results following immunofiltration and immu-
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FIGURE 3. Recovery of LTE₄ added to urine. LTE₄ (0–400 pg/mL) was added to four individual urine samples obtained from asthmatic children. Aliquots (1 mL) of urine spiked with each concentration of LTE₄ were purified by the addition of 20 µL of 50% immunoaffinity resin followed by 1 h of mixing. Quantitation was performed utilizing immunoassay #1. LTE₄ measured in unpurified urine was also determined for comparison purposes. Each individual’s samples are identified with a different symbol and connected by a line.

noaffinity purification and quantitation by immunoassay #1 is shown in Figure 6. There was a very significant correlation ($r^2 = 0.95, p < 0.0001$) between the results obtained by the two purification methods. The low y-intercept (−6) and the slope of 0.98 is also indicative of the tight relationship of the two methods.

**Evaluation of the Enzyme Immunoassay for LTE₄ Quantitation**

The effect of varying the components of the immunoassay was investigated. Two anti-leukotriene antibodies were compared in regards to sensitivity and specificity in quantifying LTE₄ in unpurified urine and in purified urine extracts. Immunoassay #1 utilized a rabbit polyclonal peptidoleukotriene antibody which was part of an assay kit commercially

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available from Cayman Chemical. Immunoassay #2 utilized a mouse monoclonal peptidoleukotriene antibody purchased from Perseptive Biosystems. Both assays utilized a peptidoleukotriene acetylcholinesterase tracer.

Both enzyme immunoassays generated standard curves with $r^2$ greater than 0.96. However, immunoassay #1 was found to be 2–4 times more sensitive than the other immunoassay. Unpurified urine samples ($n = 17$) were quantified for immunoreactive LTE$_4$ by both immunoassays. Although a significant correlation was found between the results from the two assays ($r^2 = 0.79, p < 0.0001$), immunoassay #1 gave consistently lower LTE$_4$ levels as indicated by the y-intercept of 232 (data not shown). These samples were also quantified by both immunoassays following purification using immunoaffinity resin. Levels of immunoreactive LTE$_4$ were appreciably lower with both immunoassays as compared with samples that were not purified, being 49–63% lower than the unpurified samples. There was an excellent correlation ($r^2 = 0.97$) between the results of the two immunoassays in measuring LTE$_4$ purified utilizing the
immunoaffinity resin (Fig. 7). However, similar to what was found with unpurified urine, the results obtained by immunoassay #1 were consistently lower as evidenced by a y-intercept of 80. This indicates that in addition to immunoassay #1 being more sensitive, it was also more specific for LTE₄ and less prone to interferences present in both unpurified urine and, to a lesser degree, urine purified using immunoaffinity resin.

The effect of the specific acetylcholinesterase tracer utilized in the immunoassay on the quantitative results was also investigated in another experiment. Urine samples were purified utilizing the immunoaffinity resin and the samples assayed twice using variations of immunoassay #1, once utilizing an LTE₄-acetylcholinesterase as tracer and a second time with an LTC₄-acetylcholinesterase tracer. The assay utilizing the LTC₄ tracer developed much quicker than the assay utilizing the LTE₄ tracer, but the quantitative results were identical (Fig. 8).

**Effect of the Amount of Utilized Resin on LTE₄ Quantitation**

Figure 1 suggests that 20–40 uL of resin should adequately bind any LTE₄ likely to be present in urine. We wished to further compare these two amounts of resin in regards to ability to bind [³H]LTE₄. Two urine
samples were utilized and spiked with 15000 dpm/mL of tritiated LTE₄ but no unlabeled LTE₄. After aliquoting (1 mL) samples into tubes, either no resin was added, 20 uL of resin added, or 40 uL of resin added. The samples were mixed for 1 h and then centrifuged to pellet the resin. The tritium in the supernatants was determined. In the supernatants to which no resin was added, 91% of the added tritium was found in the supernatants. In contrast, most of the tritium had been removed from all the samples that had received resin. In the samples receiving 20 uL of resin, the amount of tritium in the supernatant ranged from 12-18%, while in the samples receiving 40 uL of resin the amount of tritium in the supernatant was 13-14% of that initially added. This indicates that either 20 of 40 uL of resin added to 1 mL of urine was enough to bind over 82% of the LTE₄ in normal urine samples.

To determine whether the quantitative results were dependent upon the amount of resin utilized, urine samples were purified with both 20 uL and 40 uL of resin and the results compared following immunoassay. In 19 urine samples with creatinines between 0.34 and 2.12 mg/mL, there was no difference in the LTE₄ levels obtained with the two volumes of immunoaffinity resin (91 ± 6 with 20 uL vs. 95 ± 8 pg LTE₄/mL with 40 uL resin). However, in six samples in which urine creatinine was very low
(less than 0.34 mg/mL) there was a significant elevation in LTE₄ levels when 40 uL of resin was utilized [22 ± 4 with 20 uL vs. 36 pg LTE₄/mL with 40 uL of resin]. Since the previous experiment utilizing [³H]LTE₄ had indicated that there was no difference in the percentage of tritiated leukotrine bound by 20 uL compared to 40 uL of resin, these results suggest that impurities are bound to resin in the absence of leukotrienes in very dilute urine especially when high amounts (40 uL) of resin is present and that these impurities can cause falsely high levels of LTE₄ by immunoassay.

**Urinary LTE₄ Levels in Healthy Adults and Children**

Urine was collected from healthy adult nonsmokers and healthy children. LTE₄ levels were determined after purification using immunoaffinity resin (20 uL added) and quantification utilizing immunoassay #1 [Fig. 9]. Healthy adults (n = 14, three subjects had two separate urine samples measured and the average was taken) had a mean level of urinary LTE₄ of 80 ± 7 SEM pg/mg creatinine [interquartile range 67–96]. Urinary creat-
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FIGURE 8. The effect of utilizing LTE₄-acetylcholinesterase vs. LTC₄-acetylcholinesterase as the tracer used in enzyme immunoassay #1 on the quantitation of urinary LTE₄ in samples purified by immunoaffinity resin.

Urineine ranged from 0.34 to 2.12 mg/mL in these samples. This urinary LTE₄ level for healthy adults is similar to that previously reported by this laboratory for urine purified by HPLC or immunofiltration [68 \pm 4 pg/mg creatinine] [15,20]. Healthy children (n = 20) were also analyzed for LTE₄. The mean urinary LTE₄ level in these children was 103 \pm 9 SEM (interquartile range 70–136 pg/mg creatinine). Although many of the children had higher LTE₄ levels than the adults, there was no significant difference between adults and children (p = 0.07). Urinary creatinine in these children ranged from 0.33 to 2.26 mg/mL in these samples.

**Discussion**

A new commercially available peptidoleukotriene immunoaffinity resin was found to bind LTE₄ present in buffer, urine extract or urine, making it an ideal tool for improving existing methods for purification of LTE₄. A method of urinary LTE₄ immunoaffinity purification was found to quantitatively recover LTE₄ in urine extracts while having recovery of 63–76% in urine. The quantitative results utilizing this purification method correlated well with results obtained by a previously validated immunofil-
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**FIGURE 9.** Urinary LTE₄ in healthy adults and children (age 3–12 years). LTE₄ was purified utilizing immunoaffinity resin (20 μL added to 1 mL of urine), quantified using immunoassay #1 and normalized to creatinine. Levels of LTE₄ in children were not significantly higher than those in adults ($p = 0.07$).

The purification method of urine purification and showed good reproducibility. Two immunoassays were compared in regards to sensitivity and specificity of measuring peptidoleukotrienes in urine, with a commercially available assay found to be both more sensitive and more specific. This coupling of purification of urinary LTE₄ using an inexpensive immunoaffinity resin with quantitation by a sensitive and specific commercial immunoassay provides a universally available method to quantify this leukotriene metabolite in urine. General acceptance of this methodology would allow levels of urinary LTE₄ to gain a universal diagnostic clinical value.

This method of immunoaffinity purification and immunoassay quantitation is relatively easy, accurate and readily available. The easiest method of urine LTE₄ quantitation is a direct assay with no purification. We compared LTE₄ levels obtained by the direct quantitation of LTE₄ in unpurified urine with what was found after purification with immunoaffinity resin. Although there was a significant correlation between levels obtained by the two methods, the slope and y-intercept indicated the presence of substances that interfered with the assay when urine was not
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purified. Thus, direct quantification may be appropriate for relative comparison of samples, but its inaccuracy and relative insensitivity especially at low LTE₄ levels are problematic. Quantification of urinary LTE₄ without purification is also markedly affected by the antibody utilized for immunoassay. In comparing the two immunoassay antibodies utilized, one (immunoassay #1) showed much less interference and this was also observed in purified samples. These relatively low levels of impurities after immunoaffinity purification (compared to unpurified urine) makes the immunoassay utilized to quantify LTE₄ very important and can significantly influence the results.

Recently, another easy and sensitive method involving immunofiltration was described for quantitation of urinary LTE₄ [15]. The main drawback to this method was that the antibody utilized in the purification was not universally available. The immunofiltration and immunoaffinity resin purification methods were compared and found to give identical results. One of the attractive features of purification with this immunoaffinity resin is that the material is commercially available at such a reasonable cost (less than $1 per sample) that the resin does not need to be reused. Reusing the resin would have brought the possibility of contamination of material from previous samples. These advantages of utilizing immunoaffinity resin make it the method of choice for purifying urine samples.

The resin has the capacity to bind a large mass of leukotriene, but impurities that can interfere with the immunoassay can also bind. Thus, the amount of resin added needs to be enough to bind leukotrienes, but not too much as to bind too many contaminants. In healthy subjects, the measured LTE₄ levels were unaffected by whether the urine was purified with 20 or 40 uL of resin if urinary creatinine was over 0.33 mg/mL. However, in urine with very low creatinine levels (less than 0.33 mg/mL), significant impurities were bound which became magnified by normalization to creatinine. Although some interfering materials most likely co-purify with LTE₄ in all samples, this is the biggest problem when urine is dilute and creatinine levels are low. This problem is minimized by using 20 uL of resin instead of 40 uL of resin. However, it is still possible that at very low creatinine (less than 0.30 mg/mL) that leukotriene levels can still appear artificially elevated even when purification is performed using only 20 uL of resin and care must be taken in the interpretation of results from these samples. Because of this situation, at the present time we have chosen not to report urinary leukotriene levels in the few subjects with these low creatinine levels.

In addition to being useful for purifying LTE₄ in urine, this immunoaffinity resin could be useful in purifying leukotrienes in other fluids as well. Lavage fluid is a dilute fluid containing LTC₄, LTD₄, and LTE₄ at levels ranging from 1-50 pg/mL in most cases. The addition of immunoaffinity resin to this fluid could be useful in both purifying and concentrating the leukotrienes present in a sample. It is also possible to add two
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or more different immunoaffinity resins at the same time to a sample and purify both simultaneously. An example of this would be utilizing resins for peptidoleukotrienes and PGE₂ together. This method also has the advantage that the remainder of the fluid can be utilized for other purposes after the removal of the resin.

The normal level and range of urinary LTE₄ was determined in healthy adults utilizing this methodology. The normal urinary LTE₄ level was found to be 80 ± 7. This level is similar to normal levels previously measured following immunofiltration or after HPLC purification (15). Urine samples were also obtained from healthy children and LTE₄ measured following immunoaffinity purification. Reliable reference ranges have not previously been determined for children. The LTE₄ concentrations in children were found to have a wider range than in adults, with levels ranging between 35 and 185 pg LTE₄/mg creatinine. The mean level was 103 ± 9, which was not significantly higher than the level determined in healthy adults. The significance of high levels of LTE₄ found in some of the children is unclear, but indicates the need of further studies to obtain more detailed information.

Numerous laboratories have been involved in measuring urinary LTE₄ over the past 10 years predominantly for research purposes (4,5,12,17,19–27). These investigators have utilized a variety of methods to purify and quantify this metabolite such that basal levels differed greatly between laboratories. A recent review has summarized some of the main studies which have measured urinary LTE₄ (12). Basal levels have ranged from 40 pg/mg creatinine to 500 pg/mg creatinine. Despite these differences in mean basal levels in healthy subjects, consistent relative levels have been found universally in specific patients regardless of the laboratory performing the measurements. For example, aspirin-intolerant asthmatics have a higher basal level of LTE₄ excretion, which increases greatly following aspirin challenge (27–30). Asthmatics in general have a normal or slightly elevated level of urinary LTE₄, but this level rapidly increases following challenge with appropriate allergen (4,12,20,25). All of these results suggest that urinary LTE₄ levels are a general marker of inflammation (31).

Although the various different methodologies utilized to measure urinary LTE₄ are capable of discerning general trends in health and disease, the recent availability of leukotriene synthesis inhibitors and receptor antagonists makes a more universally comparable leukotriene assay very desirable. It will likely be important to be able to quantify urine LTE₄ from a patient and utilize this level to determine whether the person has normal or elevated leukotriene synthesis, potentially influencing appropriate drug therapy for the patient. It will also be important to be able to measure urinary levels of LTE₄ to confirm that inhibitors are effective. A universal clinical assay will allow this to be possible and should be a helpful diagnostic aid to physicians. This described methodology would be very suitable for such an assay.

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