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Rapid method for automated on-line extraction and fractionation of plasma leukotrienes and 12-hydroxy-5,8,10,14-eicosatetraenoic acids by reversed-phase high-performance liquid chromatography

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ABSTRACT

A method is described for automated on-line extraction and fractionation of plasma leukotrienes (LTs) and (5*Z*,8*Z*,10*E*,14*Z*)-(12*S*)-hydroxy-5,8,10,14-eicosatetraenoic acid [12(*S*)-HETE] by reversed-phase high-performance liquid chromatography (RP-HPLC). This method was utilized to assess the accuracy of leukotriene B_4 (LTB_4) and leukotriene C_4 (LTC_4) determinations obtained by direct immunoassay of guinea pig plasma. Plasma LTB_4 levels were significantly higher ($p < 0.01$) and plasma LTC_4 levels were unchanged when immunoassays were performed post *versus* pre RP-HPLC fractionation. Rapid separation, high recovery and baseline separation of LTB_4, LTC_4 and 12(*S*)-HETE, and minimal hardware requirements clearly demonstrate the general utility of this method.

INTRODUCTION

The area of research involving arachidonic acid (AA) metabolism has been actively explored due in part to the identification of several biologically active mediators of inflammation and allergy which are produced by the action of a 5-lipoxygenase. Among the agents which have been studied are the chemotactic eicosenoid, leukotriene (LT) B_4, and the cysteinyl-containing leukotrienes LTC_4, LTD_4 and LTE_4, potent bronchoconstrictor substances [1–3].

The ability to accurately identify and quantitate levels of these AA metabolites in tissues and in biological fluids by the use of reversed-phase high-performance liquid chromatographic (RP-HPLC) fractionation followed by immunological, biological and physiochemical characterization has allowed many laboratories to examine and elucidate the various pathways involved in AA metabolism and to determine the specificity of agents that modulate these pathways [4–10].

Due to potential cross-reactivity of AA metabolites (*e.g.* LTB_4 and 12-hydroxy-5,8,10,14-eicosatetraenoic acids (12-HETES) [11]) as well as interference by other unknown constituents, it becomes necessary to validate eicosenoid data which are obtained by direct immunological analysis of experimentally derived biological fluids. Procedures have been developed for the rapid separation of major AA metabolites contained in cell-free supernatants derived from lung tissue and polymorphonuclear leukocyte experiments (*e.g.* refs. 5–7). Some methods [5,7] require a manual pre-extraction step for sample concentration prior to RP-HPLC, making accurate

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quantitation of a large number of samples both cumbersome and time-consuming. Richmond *et al.* [6] incorporated an on-line extraction step involving a Brownlee cartridge reversed-phase precolumn followed by separation over an analytical ODS column. This 70-min procedure automates the concentration and clean-up of large (15–20 ml) volumes of biological fluid prior to fractionation and UV analysis, and can be modified to accommodate smaller volumes. However, plasma cannot be fractionated with this system since precipitated protein would block the loading column. A method for profiling lipoxygenase products by UV detection described by Borgeat *et al.* [8] although very elegant, requires several pumps and valves in addition to a standard laboratory HPLC system, making this method relatively complex and expensive to set up. A fully automated system for extraction and HPLC fractionation of 10-ml human urine samples by initial clean-up and concentration over a C_8 cartridge (each sample was extracted over a fresh cartridge) followed by separation over a C_{18} analytical column was presented by Nicoll-Griffith and Zamboni [10]. Such an approach would be necessary for analysis of either relatively large volume samples or samples which have significant numbers and/or quantities of contaminating substances. The method described herein was developed for fractionating small volumes (40 μl) of methanol-precipitated plasma and requires one external pump, two solvent switching valves, and an air control valve in addition to an HP 1090M (or similar) HPLC system.

In this paper we present a new method which consists of an HPLC system, with only minimal additional hardware requirements, for rapid automated extraction and fractionation of complex biological samples prior to enzyme-linked immunosorbent assay (ELISA) quantitation of eicosenoids.

EXPERIMENTAL

Materials

[^{3}H]Leukotrienes LTB_4, LTC_4 and LTB_4 were obtained from New England Nuclear (Boston, MA, USA); [^{3}H]12(*S*)-HETE was purchased from Amersham (Arlington Heights, IL, USA). Standard solutions of unlabeled LTB_4, LTC_4, LTD_4, LTE_4 and 12(*S*)-HETE were obtained from Biomol (Plymouth Meeting, PA, USA) and the laboratory of Dr. Richard Baker (Eli Lilly and Company, Indianapolis, IN, USA).

HPLC-grade methanol was obtained from Mallinckrodt (Paris, KY, USA) and water was Milli-Q HPLC grade (Millipore, Bedford, MA, USA).

Calcimycin (I) was kindly provided by Ms. Louise Crandall (Eli Lilly and Company, Indianapolis, IN, USA). Sodium arachidonate (AA) was obtained from NuCheck Prep (Elysian, MN, USA). Ethylenediaminetetraacetic acid, tetrasodium salt (EDTA) was purchased from Sigma (St. Louis, MO, USA). Phosphoric acid was from Baker (Phillipsburg, NJ, USA).

Borosilicate autosampler vials (Hewlett Packard, Kennett Square, PA, USA) were silanized prior to use.

Sample preparation

Blood was collected into heparinized (20 U/ml) tubes from anesthetized (35 mg/kg ketamine, Aveco, Ft. Dodge, IA, USA and 10 mg/kg xylazine, Butler, Columbus, OH, USA) six-week-old male Hartley guinea pigs (Charles River, Portage, MI, USA). Aliquots of 1 ml were incubated (in duplicate) for 5 min at 37°C in the presence of 150 μM AA, then stimulated by the addition of 20 μg/ml I in 10 μl of dimethylsulfoxide (DMSO) and incubated for an additional 10 min at 37°C. The reaction was stopped by centrifugation (1500 *g*, 10 min, 5°C) and the plasma decanted. Plasma was adjusted to 60% methanol (v/v) by the addition of HPLC-grade methanol containing 0.183 μCi/ml [^{3}H]LTB_4 (174.0 Ci/mmol) and 0.22 μCi/ml [^{3}H]12(*S*)-HETE (172 Ci/mmol) to monitor recoveries. These methanol-treated plasmas were stored under argon in polypropylene vials at −32°C until assayed. Prior to RP-HPLC, precipitated plasma proteins were pelleted by centrifugation (12 000 *g*, 5 min) at 5°C.

HPLC instrumentation and hardware

The HPLC system (Fig. 1) utilized a Hewlett Packard (HP) 1090M (Hewlett Packard, Boblingen, Germany) liquid chromatography station with a refrigerated autosampler. An RP-18 Newguard-Brownlee 7-μm, 15 mm × 3.2 mm I.D. silica guard column (Brownlee Labs., Santa Clara, CA, USA) was used as a pre-column for on-line extraction of methanolic plasma. AA metabolites were separated over a Beckman Ultrasphere octadecylsilica (ODS), 5-μm, 25 cm × 4.6 mm I.D. column (Beckman Instruments, Fullerton, CA, USA). An Eldex Model B-100-S 5000 maximum p.s.i. precision metering pump (Eldex Labs., San Carlos, CA, USA) was used to supply solvents to regenerate and re-equilibrate the guard column. Two Rheodyne Model 5301 three-way slider valves (SV1 and SV2) (Rheodyne, Cotati, CA, USA) with Model 5300 pneumatic actuators were controlled by a Rheodyne Model 7163, 24-V D.C. dual solenoid-operated air control valve which was in turn operated by two external HP 1090M 24-V switches. (The slider valves determine which solvent is pumped by the Eldex pump and are also involved in solvent recovery.) A Rheodyne Model 7030P column switching valve (CSV) is responsible for selection of an HP 1090M solvent path which either includes both pre-column and analytical column or analytical column only with the pre-column in an

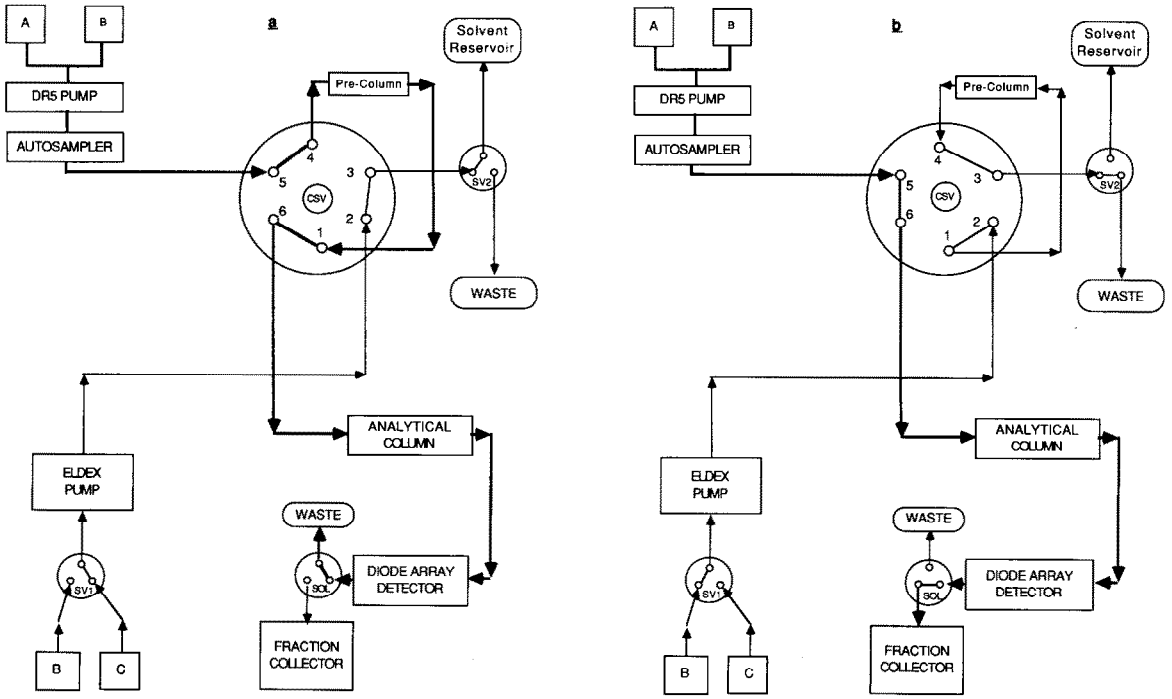

Fig. 1. Automated apparatus for on-line pre-column clean-up and fractionation of lipoxygenase products (LP) in plasma. (a) The sample is injected and pumped (heavy arrows) using the HP 1090M DR5 pump and A–B (40:60, v/v) as solvents (see Table I for composition of buffers) through the pre-column and onto the C_{18} analytical column. The PSV-100 solenoid valve (SOL) controlled by the fraction collector diverts solvents to waste. The Eldex pump, which must operate continuously during analysis, pumps solvent C (selected by three-way slider valve; SV1) through three-way slider valve 2 (SV2) to a solvent recovery reservoir to conserve solvent. (b) The column-switching valve (CSV) is activated, placing the pre-column into an external loop which reverses the flow of solvent through this column (thin arrows). The DR5 pump directs A–B (20:80, v/v) to the analytical column which elutes LP to the diode-array detector and the fraction collector which is controlled by an HP 1090M contact closure. When fractions are not being collected, the SOL directs solvent to waste. Following fraction collection, the analytical column is regenerated with A–B (5:95, v/v) and re-equilibrated with A–B (40:60, v/v). SV1 changes the solvent being utilized by the Eldex pump to 100% B to elute strongly bound material, then to A–B (40:60, v/v) to re-equilibrate the pre-column. SV2 directs contaminated solvent to waste.

external solvent path (Fig. 1). Collection of fractions containing metabolites of interest was accomplished by a Pharmacia Frac-100 fraction collector (Pharmacia-LKB Biotechnology, Piscataway, NJ, USA) which was controlled by an HP 1090M circuit containing a contact closure and a Pharmacia remote control adaptor. Solvent was diverted to waste, when fractions were not being collected, by a Pharmacia PSV-100 solenoid valve (SOL) controlled by the fraction collector.

Chromatography

Solvents consisted of (A) 0.004 m*M* phosphoric acid containing 1.5 m*M* Na_4EDTA, pH 3.3, (B) methanol and (C) methanol–0.004 m*M* phosphoric acid (60:40, v/v) containing 1.5 m*M* Na_4EDTA, pH 3.3.

TABLE I

HPLC PROGRAM

This panel outlines the HP 1090M-programmed sequence of events which control the delivery and direction of flow of solvents to the columns during a typical sample fractionation. Solvent A = 0.004 m*M* phosphoric acid containing 1.5 m*M* Na_4EDTA, pH 3.3; solvent B = methanol; solvent C = B–A (60:40, v/v). Solvent C is contained in an external reservoir and is pumped through the pre-column during pre- and post-run by the external Eldex high-pressure pump (Fig. 1).

Time (min)	Event
0.01	Flow-rate, 1.8 ml/min
0.01	Solvent A–solvent B, 40:60 (v/v)
0.01	Contact 1, on (cooling autosampler)
0.01	Contact 2, on (60:40 to pre-column)
0.01	Contact 3, off (external 60:40 goes to waste)
0.01	Column, 1 (pre-column in solvent path)
0.02	Contact 4, on (stop fraction collection)
2.00	Solvent A–solvent B, 40:60 (v/v)
2.00	Column, 0 (pre-column in external solvent path/ reverse flow)
2.10	Solvent A–solvent B, 20:80 (v/v)
4.30	Contact 4, off (fraction collection)
8.30	Contact 4, on (stop fraction collection)
9.01	Contact 2, off (methanol to pre-column)
11.00	Contact 4, off (begin fraction collection)
13.00	Contact 4, on (stop fraction collection)
16.00	Solvent A–solvent B, 20:80 (v/v)
16.50	Solvent A–solvent B, 5:95 (v/v)
19.00	Contact 2, on (solvent C to pre-column)
21.50	Solvent A–solvent B, 5:95 (v/v)
22.00	Solvent A–solvent B, 40:60 (v/v)
22.00	Contact 3, on (60:40 to reservoir)
Post time = 10 min (re-equilibration of analytical column)	

The C_{18} guard column was equilibrated with solvent C using an external ELDEX high-pressure pump. A 40-μl aliquot of the methanolic extracts was injected onto the guard column at a flow-rate of 1.8 ml/min (Table I). The composition of the eluting solvent allowed the metabolites of interest to pass through the guard column (Fig. 1a) and onto the analytical column in ≤ 2 min at which time the column switching valve (CSV) was activated to remove the guard column from the HP-1090 solvent path (Fig. 1b). Immediately following switching, the concentration of solvent B was increased to 80% (Table I) and 2-min fractions were collected at a flow-rate of 1.8 ml/min. Fractions were collected for 13 min. At 16.5 min the concentration of methanol was increased to 95% to elute any remaining material on the analytical column followed by re-equilibration by A–B (40:60, v/v). Total time for fractionation of the eicosenoids of interest and regeneration and re-equilibration of both columns required 32 min. During sample fractionation over the analytical column, the pre-column was prepared for the next sample by reversing the direction of solvent flow (Fig. 1b, activation of CSV) and washing with solvent C, then with 100% methanol (to remove very hydrophobic species) followed by a final re-equilibration with solvent C. Fractions were dried under vacuum using a Speed-Vac (Savant Instruments, Farmingdale, NY, USA), overlayed with argon and stored at −70°C.

Eicosenoid quantitation

LTB_4 and LTC_4 levels were determined by direct immunoassay of plasmas and appropriately matched RP-HPLC fractions using reagents obtained from Caymen (Ann Arbor, MI, USA). The dried RP-HPLC fractions were dissolved in 0.4 ml of ELISA buffer [100 m*M* potassium phosphate, pH 7.4, containing 1% sodium azide, 1 m*M* Na_4EDTA, 0.4 *M* NaCl and 0.1% bovine serum albumin (fatty-acid free)] and assayed with the same reagents as were used for the plasma assays.

RESULTS AND DISCUSSION

Initial experiments performed in our laboratory on guinea pig whole blood stimulated by AA and I suggested an age-dependent increase in production of the 5-lipoxygenase metabolite LTB_4. Further investigation of the metabolism of [^{14}C]AA in stimulated guinea pig whole blood demonstrated that the major metabolite was [^{14}C]12-HETE [12]. Due to the possibility of interference by plasma constituents or 12(*S*)-HETE [11] in our LTB_4 immunoassays and thus the necessity to confirm our initial data, the HPLC technique reported here was developed as a rapid, automated method for separation of these two eicosenoids from plasma.

The elution profile (Fig. 2) illustrates the separation of LTB_4, LTC_4, LTD_4, LTE_4 and 12(*S*)-HETE obtained by using the method described above. It is apparent that LTE_4 and LTD_4 co-elute and are not well separated from LTB_4. However, cross-reactivity of LTE_4 and LTD_4 in the LTB_4 immunoassay using the reagents utilized in these experiments was insignificant (<0.01%, Caymen). Therefore, carry-over of these metabolites into the LTB_4 fraction was not addressed. A mobile phase of methanol–water (8:2, v/v) containing 0.1% heptafluorobutyric acid–triethylamine, pH 3.0, as suggested by Steffenrud and Salari [7] may provide improved resolution of these leukotrienes and also decrease run time. We did not examine this solvent system in the present studies.

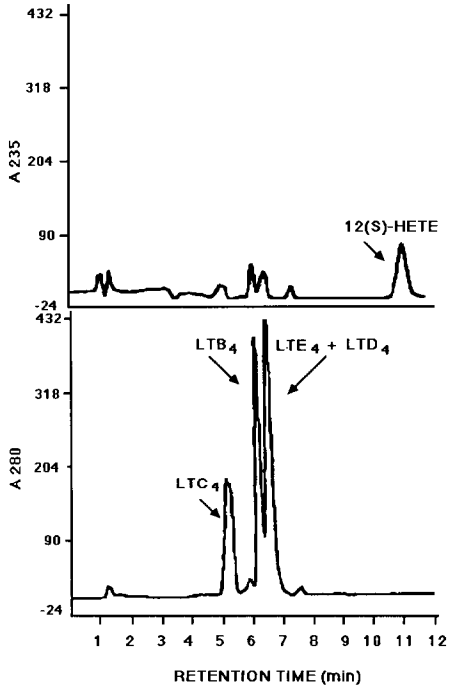

Fig. 2. Fractionation of a mixture of LTB_4, LTC_4, LTD_4, LTE_4 and 12(*S*)-HETE using the RP-HPLC program and conditions described in Fig. 1. Of each of the above eicosenoids 8 nmol were brought up in solvent C and separated over a Beckman Ultrasphere ODS 5-μm, 25 cm × 4.6 mm I.D. column. Absorbance was measured by a Hewlett Packard 1090M diode-array detector. The upper and lower panels illustrate peaks detected at 235 and 280 nm, respectively. LTE_4 and LTD_4 co-eluted in the same peak. However, LTC_4, LTB_4 and 12(*S*)-HETE were baseline-separated.

We observed improved and more consistent recoveries of leukotrienes and more stable retention times when 1.5 m*M* EDTA was included in

TABLE II

RECOVERY OF TRITIATED STANDARDS FOLLOWING RP-HPLC IN THE PRESENCE OR ABSENCE OF 40% GUINEA PIG PLASMA

[^{3}H]LTB_4 (0.53 pmol, 174 Ci/mmol, NEN), [^{3}H]LTC_4 (0.38 pmol, 39.3 Ci/mmol, NEN) and [^{3}H]12(*S*)-HETE (0.11 pmol, 172 Ci/mmol, Amersham) were placed in polypropylene microcentrifuge tubes along with identical unlabeled eicosenoids (3–5 μg of LTB_4 and LTC_4 and 1.4 ng of 12(*S*)-HETE) and evaporated under vacuum. A 200-μl volume of heparinized control guinea pig plasma or phosphate-buffered saline was added to each tube and vortex-mixed. HPLC-grade methanol was then added to each tube so that the final concentration of methanol was 60% (v/v). This mixture was vortex-mixed and placed in a −32°C freezer for 1 h to precipitate proteins, removed from the freezer and immediately spun for 2 min at 4°C at 12 000 *g* in a Sorvall Microspin 24S microcentrifuge. Supernatants were carefully recovered so as not to disturb the pellet and transferred into silanized glass micro-autosampler vials. A 75-μl aliquot was injected using the protocol described in Fig. 1. Fractions of 1.8 ml were collected and radioactivity was quantitated (n = 3, mean ± S.E.M.).

Eicosenoid	Plasma	Recovery (%)
LTB_4	−	88.7 ± 0.7
LTB_4	+	83.0 ± 1.0
LTC_4	−	81.9 ± 0.3
LTC_4	+	90.4 ± 1.2
12(*S*)-HETE	−	73.7 ± 2.6
12(*S*)-HETE	+	87.0 ± 0.1

the aqueous buffer as suggested by Peters *et al.* [5]. It was imperative that the peaks did not drift since a typical experiment consisted of up to thirty consecutive samples which were automatically injected, fractionated and collected during specific time intervals. The solvents used in the separation and column regeneration systems utilized in the method described herein resulted in highly reproducible chromatographic profiles.

Validation of both the extraction method for sample preparation prior to RP-HPLC and also the actual HPLC fractionation method was accomplished by performing the experiments described in Table II. These studies provided information on the recovery of eicosenoids following extraction and fractionation in the presence or absence of guinea pig plasma. Inclusion of both ^{3}H-labeled and unlabeled eicosenoids allowed visual monitoring of eicosenoid elution times in addition to quantitation of recovery by liquid scintillation. The recoveries for control injections of [^{3}H]LTB_4, [^{3}H]LTC_4 and [^{3}H]12(*S*)-HETE were 88.7 ± 0.7, 81.9 ± 0.3 and 73.7 ± 2.6%, respectively (mean ± S.E.M., n = 3). The recoveries [83.0 ± 1.0, 90.4 ± 1.2 and 87.0 ± 0.1%, respectively (mean ± S.E.M., n = 3)] of these tritiated eicosenoids remained excellent following extraction in the presence of control guinea pig plasma.

Aliquots of plasma which were fractionated by RP-HPLC and then re-assayed for immunoreactive LTB_4 produced values which were four-fold larger [36.04 ± 7.47 *versus* 8.83 ± 1.81 ng/ml, respectively (mean ± S.E.M., n = 5)] than those obtained by direct immunoassay of the plasma (Fig. 3). LTC_4 immunoassays performed either directly on plasma or on appropriately matched RP-HPLC fractions demonstrated that there was no significant difference [4.12 ± 0.82 *versus* 4.33 ± 0.92 ng/ml, respectively (mean ± S.E.M., n = 5)] between these two assay methods for the quantitation of LTC_4. Presumably, the unfractionated plasma contains an agent(s) which interferes with the LTB_4–antibody reaction in the immunoassay but which can be removed by fractionation.

In conclusion, a novel method is presented for automated, rapid clean-up and fractionation of plasma prior to quantitation of eicosenoids by immunoassay. This procedure is useful in the processing of large numbers of samples since it requires little hands-on manipulation, therefore increasing sample throughput and decreasing experimental error. Through simple modification of the HP 1090M program (Table I and Fig. 1) and solvents supplied to the system one can perform a wide variety of sample clean-up, concentration and fractionation procedures for compounds (or metabolites) in complex biological matrices.

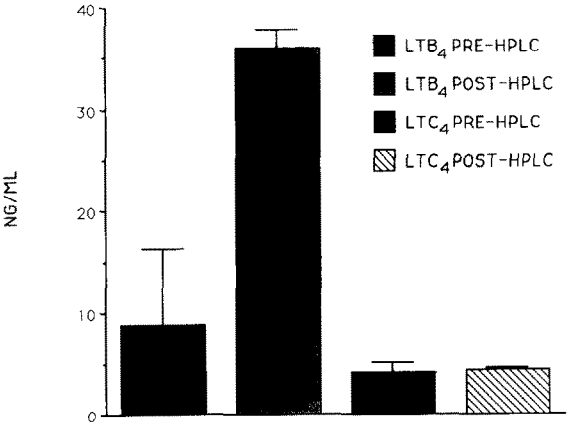

Fig. 3. Immunoassay of guinea pig plasma LTB_4 and LTC_4 before and after fractionation by RP-HPLC. Blood from one- to two-month-old guinea pigs was collected, stimulated with AA and I, and processed as described in the Experimental section. Immunoassays were performed either directly on plasma or on the RP-HPLC fractions (from the same animals) co-eluting with either [^{3}H]LTB_4 or [^{3}H]LTC_4. There was no significant difference related to the method of sample preparation for LTC_4 [pre-HPLC = 4.12 ± 0.84 ng/ml, post-HPLC = 4.33 ± 0.92 ng/ml (mean ± S.E.M., n = 5)]. Post-RP-HPLC quantitation of LTB_4 by immunoassay revealed significantly (p = 0.0076) more LTB_4 than was measured by direct immunoassay of plasma [36.04 ± 7.47 *versus* 8.83 ± 1.81 ng/ml, respectively (mean ± S.E.M., n = 5)]. The accuracy of the RP-HPLC extraction/fractionation method was confirmed by the LTC_4 data since similar amounts of LTC_4 were detected both before and after fractionation. The LTB_4 data suggest that guinea pig plasma interferes with the accurate evaluation of LTB_4 levels by the immunoassay reagents used in these experiments.

REFERENCES

1 R. A. Lewis and K. F. Austin, *Nature*, 293 (1981) 103.
2 H. R. Morris, G. W. Taylor, P. J. Piper and J. Tippins, *Nature*, 285 (1980) 104.
3 J. H. Fleisch, P. J. Calkins and D. S. Hooker, *Biochem. Pharmacol.*, 27 (1978) 2119.
4 P. Needleman, J. Turk, B. A. Jakschik, A. R. Morrison and J. B. Lefkowith, in P. D. Boyer, I. B. Dawid, A. Meister and C. C. Richardson (Editors), *Ann. Rev. Biochem.*, Annual Reviews, Palo Alto, 1972, p. 69.
5 S. P. Peters, E. S. Schulman, M. C. Liu, E. C. Hayes and L. M. Lichtenstein, *J. Immunol. Methods*, 64 (1983) 335.
6 R. Richmond, N. C. Turner, N. Maltby, D. Heavey, J. Vial, C. T. Dollery and G. W. Taylor, *J. Chromatogr.*, 417 (1987) 241.
7 S. Steffenrud and H. Salari, *J. Chromatogr.*, 427 (1988) 1.
8 P. Borgeat, S. Picard, P. Vallerand, S. Bourgoin, A. Odeimat, P. Sirois and P. E. Poubelle, *Methods Enzymol.*, 187 (1990) 98.
9 W. S. Powell, in C. Benedetto, R. G. McDonald-Gibson, S. Nigam and T. F. Slater (Editors), *Prostaglandins and Related Substances — A Practical Approach*, IRL Press, Oxford, 1987, p. 75.
10 D. Nicoll-Griffith and R. Zamboni, *J. Chromatogr.*, 526 (1990) 341.
11 F. Carey, K. H. Forder, K. H. Gibson and D. Haworth, *Prost. Leuk. Essen. Fatty Acids*, 36 (1989) 57.
12 S. M. Spaethe, C. L. Scaife, P. A. Pechous and E. L. VanAlstyne, *Biochem. Pharmacol.*, 43 (1992) 837.