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SINGLE-STEP PROCEDURE FOR THE EXTRACTION AND PURIFICATION OF LEUKOTRIENES B_4, C_4 AND D_4

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SUMMARY

A rapid and simple method for the on-line concentration and high-performance liquid chromatographic (HPLC) purification of the leukotrienes in good yield from biological fluids is described. Readily available antisera are used in conjunction with this system to give a specific and sensitive assay for leukotrienes B_4, C_4 and D_4 with sub-nanogram limits of detection. Tritium-labelled leukotrienes are used as internal standards, both to locate the leukotrienes post-HPLC and to accurately determine recoveries.

INTRODUCTION

The leukotrienes (LTs) are a family of biologically active lipids derived from arachidonic acid by the action of a 5-lipoxygenase [1]. They have been the subject of intense interest because of their profound biochemical effects produced at picomolar concentrations. The family consists of the dihydroxyeicosanoid LTB_4, a potent chemotactic agent [2], and the peptidolipid leukotrienes LTC_4, LTD_4 and LTE_4, which are responsible for the potent smooth muscle contractile effect of slow-reacting substance of anaphylaxis (SRS-A) [3,4].

In order to delineate the pathophysiological role of these species in man, an accurate and quantitative estimate is required of their formation in diseases such as asthma, where the leukotrienes are believed to act as mediators of inflammation. There are a number of assays based on the biological [5], immunological

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[6–10] and physicochemical [11] properties of the leukotrienes. To maximise specificity, a high-resolution chromatographic step, such as high-performance liquid chromatography (HPLC) is usually required prior to analysis, with the consequent losses of leukotrienes due to handling.

We have developed a high-yield, one-step extraction–purification system for the leukotrienes, and coupled it with two currently available antisera to obtain specific and sensitive assays for LTB_4, LTC_4 and LTD_4 in complex biological matrices. Our methodology is reported here, together with assay data obtained from two model systems known to generate leukotrienes.

EXPERIMENTAL

Reagents and chemicals

Methanol was HPLC grade from Rathburn Chemicals (Peebleshire, U.K.) and water was Millipore-Q quality. ^{3}H-Labelled LTB_4, LTC_4 and LTD_4 were purchased from New England Nuclear (Dupont, Stevenage, U.K.) (specific activities of 40.3 Ci/mmol). Additional [^{3}H]LTC_4 was obtained from Amersham International (Amersham, U.K.) (specific activity of 45.1 Ci/mmol). Authentic leukotrienes employed as standards were generous gifts of Dr. J. Rokach, Merck-Frosst Canada (Pointe-Claire/Dorval, Canada). The rabbit antisera to LTC_4 and LTD_4 were a generous gift of Dr. E.C. Hayes, Department of Immunology and Inflammation Research, Merck Institute for Therapeutic Research (Rahway, NJ, U.S.A.) and the LTB_4 radioimmunoassay kit was obtained from Amersham International. Desferal was obtained from Ciba-Geigy, polyvinylpyrrolidine (PVP-40), ionophore A23187, activated charcoal and phenylmethylsulphonyl fluoride were obtained from Sigma (Poole, U.K.). All other chemicals were of Analar grade.

Samples

Guinea pigs (male, Dunkin Hartley, 300–500 g) were sensitised with ovalbumin [12]; between two and four weeks post-sensitisation, the animals were killed and their lungs perfused with oxygenated Tyrode's solution. The perfused lungs were challenged with ovalbumin and the perfusate was collected on ice. Radiolabelled leukotrienes (7500 dpm) were added, the perfusate was lyophilised and stored at −80°C until required.

Blood samples (25 ml) were taken from drug-free volunteers by venepuncture and either used directly or after stimulation with ionophore A23187 (25 μg/ml) at 37°C for 30 min. It was mixed with an equal volume of inhibitory mixture to prevent ex vivo metabolism and biosynthesis. The mixture in isotonic saline contains L-cysteine, nordihydroguaritic acid, indomethacin and serine borate to give final concentrations in diluted whole blood of 10 mM, 50 μM, 2.8 μM and 10 mM, respectively. For the ionophore challenge study, 2500 dpm of ^{3}H-radiolabelled LTB_4 were added per ml of blood either on collection or immediately following ionophore stimulation; the mixture was centrifuged (1000 g, 15 min) and the supernatant was stored at −80°C until required.

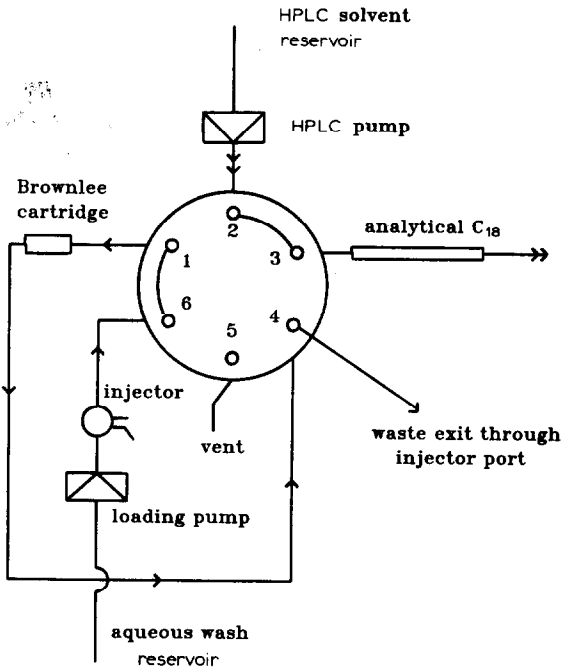

Fig. 1. Diagramatic representation of a Rheodyne 7125 injector, modified for on-line one-step extraction and purification of the leukotrienes. Biological fluids are pumped through port No. 6 and routed through a Brownlee cartridge containing MCH-10, with aqueous waste eluting through port No. 4; HPLC solvent passes through port No. 2 and onto the HPLC column via port No. 3. When the sample has been loaded and washed with two volumes of 0.04% aqueous acetic acid (buffered to pH 3.8 with ammonia), port No. 2 is linked to port No. 1, with No. 4 linked to No. 3. The HPLC solvent passes through the cartridge, eluting the leukotrienes directly onto the analytical HPLC column.

Chromatography

A Varian 5020 HPLC solvent delivery system (Varian Assoc., Walton on Thames, U.K.) was used coupled to a Model 440 UV detector (Waters Assoc., Harrow, U.K.). Crude samples were extracted using a Brownlee C_{18} cartridge (Anachem, Luton, U.K.) containing MCH-10 reversed-phase packing (Varian); this was attached in line to the analytical HPLC column (Fig. 1). A Waters 6000A pump was used as the auxiliary HPLC (loading) system. HPLC columns (25 cm×0.5 cm I.D.) were slurry-packed at 550 bar with 5-μm Hypersil ODS (Shandon Southern Products, Astmoor, Runcorn, U.K.), giving typical efficiencies of 45 000–50 000 plates per m. Standard elution conditions involved a linearly increasing methanol gradient (methanol–water–acetic acid, 57:43:0.017 to 96:4:0.0016, v/v/v) at 1 ml/min over 90 min. In later studies, oxalic acid (0.5 m*M*) was added to the time zero eluent, which was then was adjusted to pH 3.7–3.8. Bed-to-bed Shandon guard 2-cm columns dry-packed with Whatman Co:Pell (Whatman, Springfield Mill, Maidstone, U.K.) were used. After approximately twenty analyses, the system was reconditioned by injection with 100 μl of 10 m*M* aqueous oxalic acid or 0.3% (w/v) aqueous EDTA containing 0.01% (w/v) Desferal.

Immunoassay

LTB_4 was determined with a commercial immunoassay kit (Amersham International). The immunoassay for the peptidoleukotrienes is a modification of that developed by Hayes et al. [10], who kindly provided the antisera. Samples were resuspended in 0.3 ml of radioimmunoassay (RIA) working buffer (0.014 *M* sodium chloride, 0.02% sodium azide, 0.1 m*M* phenylmethylsulphonyl fluoride, 1 mg/ml PVP, 7.15 m*M* Na_2HPO_4–0.2m*M* NaH_2PO_4 pH 7.2), an aliquot was taken and liquid scintillation counted to estimate final delivery of peptidoleukotriene to the RIA. The remaining volume (0.150 ml) was added to 0.025 ml containing 25 000 dpm of [^{3}H]LTC_4 and 0.025 ml of a 1:200 dilution of rabbit antisera. This mixture was then incubated together at 4 °C for 18 h. Separation of the resultant bound and unbound ligand was achieved by adding 1 ml of ice-cold activated charcoal–dextran mixture (62.5 mg dextran per 100 ml and 62.5 charcoal per 100 ml in 0.014 *M* sodium chloride, 0.002% sodium azide, 0.01 m*M* phenylmethylsulphonyl fluoride, 0.1 mg/ml PVP, 0.715 m*M* Na_2HPO_4–0.02 m*M* NaH_2PO_4 pH 7.2) and leaving for 15 min at 4 °C. Following centrifugation at 1200 *g* for 15 min, the supernatant was decanted into scintillation vials containing 10 ml of scintillant and counted on a Beckman Tri-Carb Model 3375, employing Beckman Instagel scintillant, in 20-ml plastic scintillation vials with efficiencies in the range 37–39%. Liquid scintillation counts were processed by an unweighted four-parameter logistic curve fitting program resident on a Perkin-Elmer 3220 minicomputer. The tritiated LTB_4, LTC_4 and LTD_4 stocks were periodically examined every three months by HPLC after storage at −20 °C. The histamine content of each sample was determined when necessary [13].

RESULTS AND DISCUSSION

A number of groups have used solid-phase extraction coupled with HPLC for the purification of the leukotrienes prior to analysis [14–16]. One drawback of such systems is the requirement for solvent removal and resuspension at each stage of purifiation. This can be time-consuming and often results in lowered yields due to analyte adsorption on the surface of extraction glassware. To overcome these problems, we have developed and applied a single-step extraction and purification procedure for the peptidoleukotrienes and also for LTB_4.

On-line extraction–concentration was investigated using both Waters Guard-Pak insert cartridges and the Brownlee cartridge holder system, positioned in the loop of the Rheodyne 7125 injection valve. The reversed phases employed in the former case were Resolve™, CN and μBondapak and in the latter case Varian MCH-10 phase. Peptidoleukotrienes recoveries for the first three phases were in the range 50–70% while the Varian phase MCH-10 gave recoveries in the range 80–95%. However, although the 3-cm Brownlee cartridge packed with MCH-10 gave the higher recoveries, loading of the cartridge was characterized by relatively high back-pressure making syringe injection difficult. This was circumvented by the adaption of the Rheodyne 7125 valve to behave as a Rheodyne 7000 valve [17]. This modification involved using the 7125 valve low-pressure loop exit port (number 6) as a high-pressure aqueous solvent input port, employing an auxil-

iary HPLC pumping system. In consequence, this modified 7125 valve allowed the loop Brownlee–MCH-10 cartridge to be loaded using an HPLC pump pressurized solvent stream. The loading flow direction was reversed compared with the more conventional injection from the front of the 7125 injection valve. The use of a conventionally loaded Rheodyne 7125 injection valve fitted with a 5-ml loop in the auxiliary HPLC loading system permitted large volumes to be injected (Fig. 1).

HPLC elution conditions for the leukotrienes were optimised on a end-capped Hypersil ODS 5-μm column eluting with a methanol–water–acetic acid system. The retention times for LTC_4, LTB_4, LTE_4 and LTD_4 in this system are 29, 38, 39 and 50 min, respectively, with recoveries of 66–70% for LTC_4, 64–77% for LTD_4 and 80–88% for LTB_4. The required HPLC conditions were determined by: (i) the cross-reactivity of the available LTC_4 and LTB_4 antisera (i.e. the requirement for good separation of immunoreactive species) and (ii) the requirement for on-line column stripping, with the initial mobile phase containing sufficient organic modifier to elute leukotrienes from the extraction column (Brownlee), without affecting the chromatography on the analytical column (Hypersil). Oxalic acid (0.5 m*M*) is included in the HPLC starting buffer, together with occasional washes with 10 m*M* oxalate, to optimise peak shape and recovery [18]. Interestingly, one group has reported that neither LTC_4 nor LTD_4 elute from Hypersil ODS [19]. This was also found in this laboratory until EDTA or oxalic acid conditioning was introduced. We have occasionally supplemented the EDTA or oxalate with the highly affinic ferric ion chelator Desferal as a precaution against leukotriene inactivation or chelation by iron.

Due to the variant recoveries and chromatographic behaviour of the peptidoleukotrienes in many HPLC systems, it was essential to have unambiguous internal standards. Although prostaglandin (PG) B_2 is often used as an internal standard for peptidoleukotrienes (especially in UV detector-orientated studies) [11], it was regarded as structurally too dissimilar to the peptidoleukotrienes to be useful. Tritiated LTC_4 and LTD_4 were employed as internal standards for every processed sample. Sufficient was added to the sample (i.e. 7500 dpm of both [^{3}H]LTC_4 and [^{3}H]LTD_4, equivalent to approximately 50 pg) to give accurate recovery estimation, yet insufficient to interfere with the post-HPLC RIA.

Using the rabbit antisera to LTC_4 of Hayes et al. [10] we have made minor but significant improvements to the range and sensitivity of their published assay. These include lowering the total incubation volume whilst increasing the sample volume, replacing bovine serum albumin (BSA) with PVP in order to lower non-specific binding and using a charcoal adsorption step, thus measuring bound rather than free ligand. Using this modified assay, standard curves were constructed for LTC_4 and LTD_4 in the ranges 8–4700 and 7–3300 pg, respectively; on the basis of eleven points carried out in duplicate, 50% displacement for LTC_4 was 300 pg ($n=10$) and for LTD_4 500 pg ($n=10$). The detection limits of the immunoassay ($<10\%$ displacement) are 120 pg LTC_4 and 400 pg LTD_4. The RIA for LTB_4 was undertaken with the commercially available kit without modification.

Typically between 15 and 20 ml of the biological fluid was spiked with tritiated internal standards, centrifuged to remove cellular material, decanted and the pH

adjusted to 3.8. This was loaded onto the 5-ml loop and injected into an aqueous solvent stream (0.04%, v/v, acetic acid, pH 3.8) flowing at 3 ml/min through the Brownlee–MCH-10 C_{18} cartridge, giving typical back-pressures of 21–28 bar. Leukotrienes were eluted from the Brownlee cartridge by switching into the HPLC solvent stream and initiating the methanol gradient. The eluate from the initial Brownlee cartridge extraction passes directly onto the analytical ODS column where chromatography occurs; losses due to sample handling at this stage are thus eliminated. The recovery from crude perfusate across this multi-column system is approximately 55% for both LTC_4 and LTD_4 and 65% for LTB_4. Fractions (2 min) post-HPLC were collected and 5–10% of each 2-ml fraction was used to determine the exact elution position of each leukotriene. Four 2-ml fractions around each tritiated peptidoleukotriene peak were pooled and either lyophilised or evaporated to dryness under nitrogen; the former procedure was considered more practicable with large sample numbers. After evaporation the samples were resuspended in RIA working buffer and assayed.

Extracted standard curves were obtained for LTB_4, LTC_4 and LTD_4; a good correlation between leukotriene added and leukotriene detected (corrected for recovery with the radiolabelled internal standard) was obtained for LTD_4 and LTB_4, but for LTC_4 only ca. 40% of exogenous immunoreactive material was recovered compared with the [^{3}H]LTC_4 internal standard. On chromatographing larger quantities of LTC_4 (5 μg) through our system, no change in the physicochemical or immunological properties were noted, indicating that the problem of reduced immunoreactivity is only observed at low levels and may arise through lactonisation of LTC_4 on surfaces [20], perhaps catalysed by the γ-carboxyl group. This may affect immunoreactivity without reducing the recovery of ^{3}H radiolabel.

A series of guinea pig lung perfusates (from both sensitised and sham sensitised) were analysed using our single-step extraction–HPLC system and improved RIA; the data are shown in Table I. LTD_4 is observed, together with lower amounts of LTC_4, in the perfusate from sensitised lungs only. Using tritiated standards, we can demonstrate that there is no conversion of LTC_4 to LTD_4 or further to LTE_4 in guinea pig lung perfusate (in agreement with the very low levels of γ-glutamyltransferase in the perfusate [21]). There is a significant linear correlation between LTD_4 and histamine levels in the perfusate demonstrating the efficacy of sensitisation for each animal. A representative full immunochromatogram of guinea pig lung perfusate showing LTC_4 and LTD_4 is shown in Fig. 2. It is clear from the accompanying $A_{280\ nm}$ trace that other non-immunoreactive species are present. Thus it would clearly be impossible to quantify the leukotrienes by their UV absorbance.

A sample of whole blood was analysed for immunoreactive peptidoleukotrienes C_4 and D_4; the immunochromatogram (Fig. 3) clearly shows that there is a high background and that without HPLC analysis there is a high probability of overestimating leukotriene levels. There also appears to be a small peak of immunoreactivity coeluting with the [^{3}H]LTD_4 marker. As this is unlikely to represent circulating LTD_4, it may have arisen on collection. Following this finding, we routinely collect biological samples into an inhibitory mixture containing nordihydroguaritic acid (to inhibit post-collection generation of the leukotrienes),

TABLE I

PRODUCTION OF PEPTIDOLEUKOTRIENES AND HISTAMINE IN GUINEA PIG LUNG PERFUSATE

Peptidoleukotriene in guinea pig lung perfusate as determined by on-line extraction-HPLC-radioimmunoassay. Data is given as ng leukotriene per 90 ml perfusate, both pre- and post-antigen challenge. Animal No. 6 was sham sensitised to act as a control. For animal Nos. 1-5 there is an appreciable increase in LTD_4 formation on challenge, with small amounts of LTC_4. Histamine levels are shown for comparison.

Animal No.	LTC_4		LTD_4		Histamine	
	Pre	Post	Pre	Post	Pre	Post
1	2.5	4.6	5.4	79.8	20	3807
2	1.8	3.5	7.0	12.1	22	680
3	2.9	3.0	12.8	18.7	38	2133
4	2.7	4.7	5.4	34.3	27	3402
5	2.9	25.0	12.4	446.0	33	10800
6	1.8	2.8	7.3	4.4	34	29

together with serine borate and L-cysteine (to prevent LTC_4 metabolism and the formation of the weakly immuoreactive LTE_4 by inhibiting γ-glutamyltransferase activity and cysteinylglycinylpeptidase activity). With the inhibitory mixture present, no metabolism occurred in whole blood (Fig. 4A); without the inhibitory mixture, LTC_4 was partially converted to LTD_4 and LTE_4 in the short time required to handle the sample (Fig. 4B). To determine the extraction yield for leukotrienes in whole blood, 2 ng each of LTC_4 and LTD_4, together with ^3H-labelled internal standards, were added to 20 ml of blood. The immunochroma-

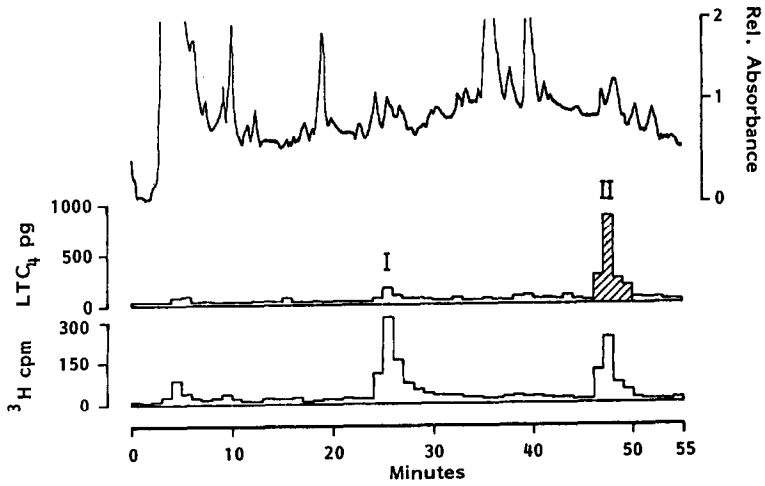

Fig. 2. Representative chromatogram of guinea pig lung perfusate following antigen challenge, showing A_{280nm} absorbance, LTC_4 immunoreactivity and radioactivity (from ^3H-labelled internal standards). LTD_4 (II) is present with smaller amounts of LTC_4 (I). It is clearly not possible to use A_{280nm} absorbance to detect the leukotrienes with any confidence.

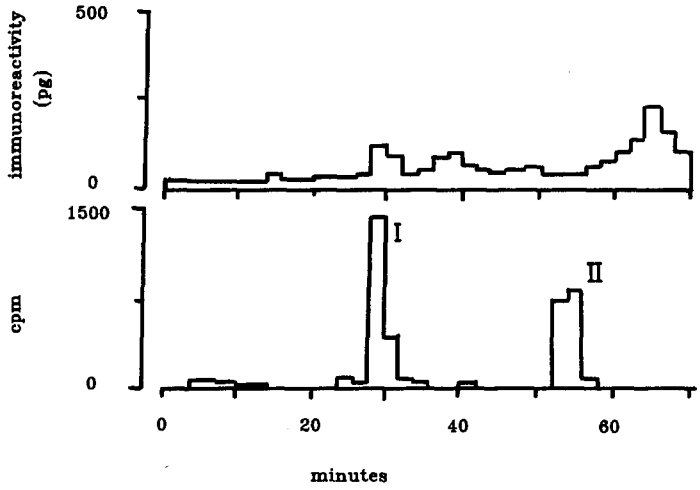

Fig. 3. Chromatogram from a sample of whole blood (20 ml, with the inhibitory mixture but without nordihydroguaritic acid) extracted and purified through the on-line stripping system. The ^{3}H-radiolabelled internal standards define the elution position for LTC_4 (I) and LTD_4 (II). Background immunoreactivity is high, with a peak in the LTC_4 position. This is unlikely to represent circulating LTC_4, and probably arose on collection. A 5-lipoxygenase inhibitor, nordihydroguaritic acid, is now included in our inhibitory mixture to prevent post collection generation of leukotrienes.

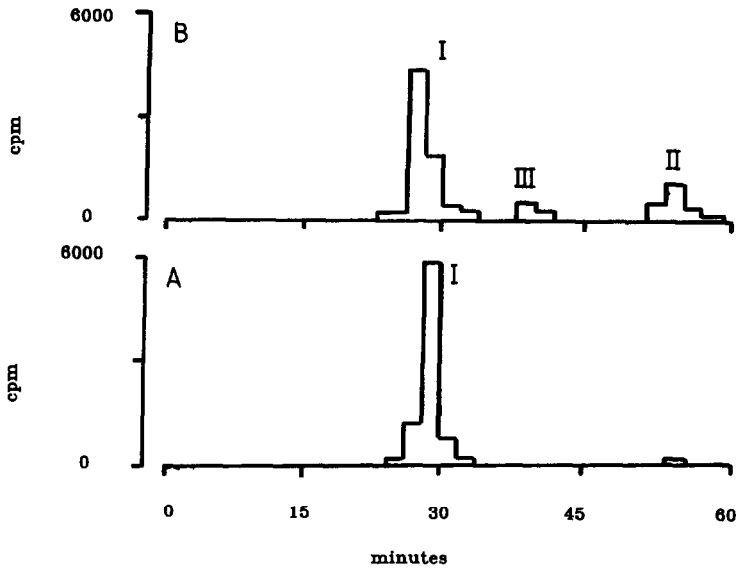

Fig. 4. Radiochromatogram of [^{3}H]LTC_4 added to whole blood, extracted and purified with the on-line stripping system. (A) In the presence of inhibitory mixture, LTC_4 (I) is not metabolised, even after storage at −80°C for eleven days. (B) With EDTA present (as an anticoagulant), but in the absence of inhibitory mixture, LTD_4 (II) and LTE_4 (III) are generated.

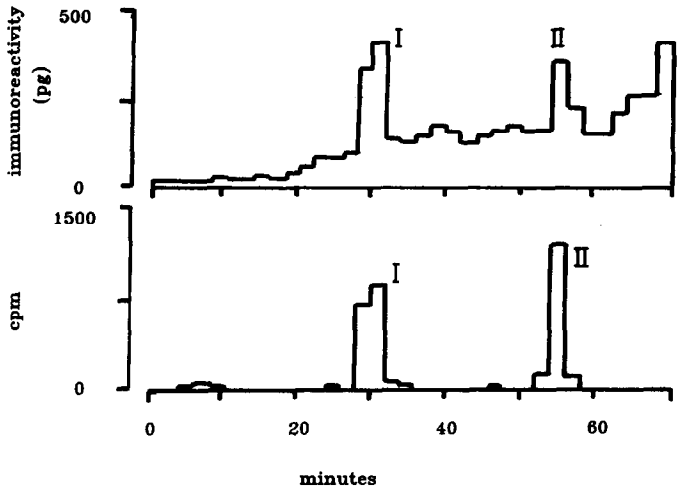

Fig. 5. Immunochromatogram from 20 ml blood, to which 2 ng each of LTC_4 and LTD_4 had been added. Recoveries of the radiolabelled internal standards were 73 and 55% for LTC_4 (I) and LTD_4 (II), respectively.

togram (Fig. 5) shows good recovery for the internal standards and measurable immunoreactivity for LTC_4 and LTD_4 above the background.

Using our extraction–analysis system, LTB_4 production from ionophore-stimulated blood was also measured. A representative immunochromatogram is shown in Fig. 6. We are currently using this ex vivo method to monitor the efficacy of 5-lipoxygenase inhibitors in man [22].

We have optimised our extraction–HPLC system on a Brownlee cartridge con-

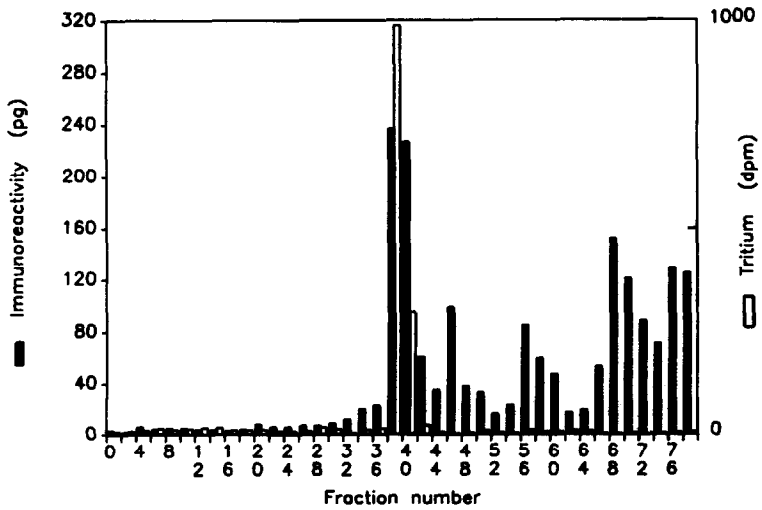

Fig. 6. Immunochromatogram of LTB_4 generated by ionophore treatment of whole blood. The major peak of immunoreactivity coelutes with the [^{3}H]LTB_4 internal standard (open bars). There is appreciable immunoreactivity in later fractions (cf. Fig. 5), emphasising the importance of HPLC purification prior to immunoassay.

taining MCH-10 coupled to a 5-μm Hypersil ODS column leading to improved recoveries. The value of a single-step extraction protocol is clear; the procedure is rapid: 70 min from crude perfusate to HPLC-pure peaks; futher, sample handling, and consequent losses due to surface adsorption, is minimised. Pickett and Douglas [20] have used a similar approach to extract and purify platelet 12-hydroxyeicosatetraenoic acid (12-HETE) in good yield; they demonstrated with radiolabelled species that their system was suitable for the analysis of PGE_2, LTC_4 and 5-HETE. With single-step extraction–purification systems, there is no limit to the volume of simple biological fluids that may be analysed with this system – indeed, we have loaded up to 100 ml of guinea pig lung perfusate with good recovery and chromatography (data not shown). Proteinaceous fluids such as blood are limited to about 5 ml, otherwise the loading column becomes blocked with precipitated protein.

We are, however, still limited to measuring LTB_4 and/or LTC_4 and LTD_4 as the cross-reactivity of the available antisera with LTE_4 are low. This can be a problem as, for example, LTE_4 is rapidly generated in blood [23], and in man it was found in urine following a bolus dose of LTC_4 [24]. Recently an RIA kit more suitable for LTE_4 has become available (Amersham International) and should prove useful in conjunction with our extraction–HPLC system to monitor LTE_4 levels in plasma and urine in man.

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