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DETERMINATION OF LEUKOTRIENES AND PROSTAGLANDINS IN [^{14}C] ARACHIDONIC ACID LABELLED HUMAN LUNG TISSUE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND RADIOIMMUNOASSAY

FREDERIK J. ZIJLSTRA* and J. ERIC VINCENT

Department of Pharmacology, Medical Faculty, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam (The Netherlands)

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SUMMARY

A liquid chromatographic method for the determination of ^{14}C-labelled prostaglandins, leukotrienes and other lipoxygenase products formed by human lung tissue is described. In this paper we report our problems identifying these substances when ^{3}H- or ^{14}C-labelled compounds are compared with measurements of the mass by absorption or radioimmunoassay. Furthermore, some preliminary results of [^{14}C]arachidonic acid labelled human lung tissue, stimulated by the Ca-ionophore A23187, show that, of the lipoxygenase products, mostly leukotriene B_4 like compounds are formed and less leukotriene C_4, E_4 and D_4. Relatively large amounts of hydroxyeicosatetraenoic acids are present. The main cyclooxygenase products are thromboxane B_2, 6-ketoprostaglandin $F_{1\alpha}$ and prostaglandin D_2.

INTRODUCTION

The biologically active leukotrienes (LTs) are formed from arachidonic acid (AA). Several of these substances induce slow contractions in smooth muscles. The effects of the LTs on peripheral and central airway functions of pulmonary tissue have also been described [1]. Furthermore, it has been reported that the addition of leukotriene C_4 (LTC_4) and D_4 (LTD_4) induces thromboxane A_2 (TxA_2) release from guinea pig isolated perfused lungs [2, 3]. Both the contraction of lung parenchymal strips and the TxA_2 release could be inhibited by the β-adrenoceptor agonist isoprenaline [4]. In previous work, a comparison was made between the contractile activities and TxA_2 release of human, porcine and guinea pig lung parenchymal strips after the application of LTC_4 and LTD_4 [5]. The results indicate that both the contractile activity of LTs on human lung strips and the TxA_2 release were rather low in com-

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parison with the guinea pig lung strip. In cultured endothelial cells from human umbilical vein, however, LTC_4 promotes prostacyclin synthesis [6]. Recently, it has become evident that human alveolar macrophages produce leukotriene B_4 (LTB_4) [7], and it was also shown that human peritoneal macrophages synthesize LTB_4 and LTC_4 [8]. Further, Dahlén et al. [9] showed that allergen challenge of chopped human lung tissue elicits contraction that correlates with the release of both LTC_4, LTD_4 and leukotriene E_4 (LTE_4) and prostaglandins.

In this paper we describe an extraction procedure and high-performance liquid chromatographic (HPLC) techniques for the separation of cyclooxygenase and lipoxygenase products with comparatively high recoveries and discuss some of the problems concerned with the identification of these substances formed from [^{14}C]AA-labelled and Ca-ionophore-triggered chopped human lung tissue.

EXPERIMENTAL

Apparatus

A 1082B high-performance liquid chromatograph (Hewlett-Packard) was used, consisting of double-head pump, temperature-controlled column compartment, variable-volume injector and variable-wavelength detector. The Superrac fraction collector (LKB, Sweden) was connected to this apparatus and used as an automatic sampling system. Radioactivity in the labelled fractions was counted in a 3255 Tricarb liquid scintillation counter (Packard, Brussels, Belgium).

Chemicals

LTB_4, LTC_4 and LTD_4 were gifts of Dr. J. Rokach (Merck Frosst, Canada). Ca-ionophore A23187 was obtained from Hoechst (Calbiochem-Behring, U.S.A.), reduced glutathione from ICN (Cleveland, OH, U.S.A.) and prostaglandins D_2 (PGD_2), E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) from Sigma (U.S.A.). 6-Keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and thromboxane B_2 (TxB_2) were gifts of Dr. J.B. Smith (Philadelphia, PA, U.S.A.). Siliclad® was obtained from Clay Adams (Becton Dickinson, U.S.A.). Tetrahydrofuran, methanol, acetonitrile, benzene and acetic acid were all of analytical grade from E. Merck (Darmstadt, F.R.G.). Picofluor-15 (Packard) was used as premixed scintillation cocktail.

Radiochemicals

[1-^{14}C]AA, 5-D-[5,6,8,9,11,12,14,15-^{3}H(n)]hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), 12-L-[5,6,8,9,11,12,14,15-^{3}H(n)]HETE and 15-L-[5,6,8,9,11,12,14,15-^{3}H(n)]HETE were purchased from New England Nuclear. All other radiolabelled compounds mentioned below were obtained from the Radiochemical Centre (Amersham, U.K.): [5,6,8,9,11,12,14,15-^{3}H(n)]LTB_4, [14,15-^{3}H(n)]LTC_4, [14,15-^{3}H(n)]LTD_4, 6-keto-[5,8,9,11,12,14,15-^{3}H(n)]-$PGF_{1\alpha}$, [5,6,8,9,11,12,14,15-^{3}H(n)]TxB_2, [5,6,8,11,12,14,15-^{3}H(n)]PGE_2, [5,6,8,11,12,14,15-^{3}H(n)]$PGF_{2\alpha}$, [5,6,8,9,12,14,15-^{3}H(n)]PGD_2, [1-^{14}C]PGE_2 and [1-^{14}C]$PGF_{2\alpha}$. The purity of the radiochemicals was shown to be greater than 97%.

Materials

Sep-Pak C_{18} and Sep-Pak silica cartridges and HPLC-solvent filters HA (0.45 μm) and FH (0.5 μm) were obtained from Waters Assoc.; prepacked HPLC columns Nucleosil $5C_{18}$ and Zorbax $BPtmC_8$ (each 250 × 4.6 mm) were from Chrompack (Middelburg, The Netherlands).

Antisera

Anti-TxB_2, anti-$PGF_{2\alpha}$, anti-PGE_2 and anti-PGD_2 were obtained from l'Institut Pasteur (Paris, France); anti-6-keto-$PGF_{1\alpha}$ was obtained from Seragen (Boston, MA, U.S.A.). Cross-reactivities are given in Table I.

TABLE I

CROSS-REACTIVITIES OF COMMERCIALLY OBTAINED ANTIBODIES

Data are expressed in per cent at 50% B/B_0 (B = bound).

Compound	Antibody				
	6-Keto-$PGF_{1\alpha}$	TxB_2	$PGF_{2\alpha}$	PGE_2	PGD_2
6-Keto-$PGF_{1\alpha}$	100	—	0.04	<0.01	0.01
$PGF_{1\alpha}$	7.8	—	12.0	0.01	0.01
$PGF_{2\alpha}$	2.2	0.1	100	0.11	0.04
6-Keto-PGE_1	6.8	—	—	0.16	—
PGE_1	0.7	—	0.03	10.7	0.01
PGE_2	0.6	0.1	0.03	100	0.01
PGA_1	<0.01	—	<0.01	0.04	—
PGA_2	<0.01	0.1	<0.01	0.3	—
PGD_2	<0.01	0.2	3.0	<0.01	100
PGD_1	—	—	<0.4	<0.01	78
TxB_2	<0.01	100	<0.01	<0.01	1.2
13,14-Dihydro-15-keto-PGE_2	<0.01	0.1	—	0.6	0.01
13,14-Dihydro-15-keto-$PGF_{2\alpha}$	<0.01	0.1	<0.01	<0.01	0.01
13,14-Dihydro-PGE_2	—	—	—	2.1	—
6-Keto-PGE_2	—	0.1	<0.01	13.2	—

Human lung tissue

Human lung tissues were obtained from adults [5]. Lobectomy was performed on account of tumours. The premedication consisted of 0.25 mg of atropine and 10 mg of Opial®. During the operation, the following agents were administered: Fentanyl® (0.5 mg), pancuronium bromide (Pavulon®, 6 mg), thiopental (Pentotal®, 150 mg) and 3 g of cephalothin (Keflin®). Parenchymal tissue of the outer parts was used in our experiments.

Method

Human lung tissue (10 g) from which the lung membrane had been removed was cut into slices. The slices were chopped in a McIlwain tissue chopper and divided in portions of 0.5 × 0.5 mm. The whole fraction was washed three times with Krebs–Henseleit buffer (20 ml of buffer, 5 min, 400 *g*) in a polypropylene 50 ml tube (Falcon®). Then 20 ml of Krebs–Henseleit buffer were added and the tube was placed in a water-bath of 37°C on a magnetic stirrer (900 rpm). Through a thin pipette, the sample was continuously gassed with a

mixture of 95% O_2 + 5% CO_2. Thereafter, 10 μCi of [1-^{14}C]AA (55 mCi/mmol), glutathione (final concentration 2 m*M*) and 100 μg of Ca-ionophore A23187 (dissolved in 100 μl of ethanol) were added. At the end of the 10-min incubation, [^{3}H]LTs and [^{3}H]PGs were added and the homogenate was centrifuged (10 min, 1400 *g*, 4°C). The pellet was washed once, and the combined supernatant centrifuged (90 min, 30,000 *g* average, 4°C) to separate the cells and small particles. The clear incubation supernatant was then applied to a Sep-Pak C_{18} cartridge and the effluent was placed on a Sep-Pak silica cartridge. (The C_{18} cartridge was prewashed with 10 ml of methanol and 10 ml of distilled water; the silica cartridge was prewashed with 10 ml of methanol and 100 ml of water [10].) The sample was eluted with 2.5 ml of methanol on each column; these eluates were combined and evaporated to dryness with a gentle stream of nitrogen at 40°C. Thereafter, the dried sample was dissolved in 1 ml of solvent A (tetrahydrofuran—methanol—water—acetic acid, 25:30:45:0.1, v/v, adjusted to pH 5.5 with ammonium hydroxide), filtered and kept in a siliconized micro-vial.

Chromatographic system

Reversed-phase HPLC of LTs and other lipoxygenase products was carried out on a Nucleosil 5 C_{18} column, using solvent system A. Mobile phases were filtered by vacuum filtering through a Millipore filter and degassed with helium [11, 12]. The flow-rate was 0.9 ml/min and the absorption was measured at 280 nm. Prior to use, the system was washed with approx. 15 ml of water, thereafter with approx. 30 ml of a 2% (w/v) EDTA solution in water, and rewashed with water [10]. The column was equilibrated with the mobile phase A at an oven temperature of 37°C. Fractions were collected for scintillation counting. After each run (90 min) the column was rinsed for at least 30 min because of contamination with Ca-ionophore, which elutes after approx. 115 min.

Reversed-phase HPLC of PGs was performed on a Zorbax C_8 column. This solvent system (B) contained acetonitrile—benzene—water—acetic acid (24:0.2:0.1:76, v/v). The flow-rate of this eluent was 2.0 ml/min. From the contents of each collected fraction, 50 μl were taken and kept at 4°C for the radioimmunoassay (RIA) of the PGs. The main fraction was immediately used for ^{14}C and ^{3}H counting. The column was rinsed with acetonitrile for 30 min after each sample to elute the lipoxygenase products.

Radioimmunoassay of prostaglandins

Reagents were equilibrated to room temperature before use. After the addition of standards (range 0—500 pg) and diluted samples, [^{3}H]PG and antibody were added. At the end of the incubation (2 h at room temperature and 18 h at 4°C), charcoal suspension was added. The tubes were allowed to stand for 15 min at 4°C and centrifuged for 10 min at 1400 *g*. The supernatants were decanted and mixed with 6 ml of scintillation fluid. The risk of cross-reactions was negligible when RIA was performed after HPLC (cross-reactivities and specifications are given in Tables I and II).

TABLE II

SPECIFICATIONS OF VARIABLE CONDITIONS IN THE RIAs

Immunogen	Amount of added tracer		Bound/total (%)	Non-specific binding (%)
	dpm	pg		
6-Keto-$PGF_{1\alpha}$	16,000	18.4	39.7	9.2
TxB_2	15,000	18.5	57.9	1.9
$PGF_{2\alpha}$	8,500	9.2	17.0	4.5
PGE_2	12,500	13.0	40.1	3.9
PGD_2	8,500	10.7	34.3	7.4

Quantitative evaluation

The settings for double-labelled scintillation counting were such that there was no spillover of radioactivity of ^3H into the ^{14}C channel. Calculations of dpm were carried out using quenched standard sets by a computer (Digital, PDP 11/70). For daily analysis, a plotting system was programmed in order to obtain data of total counts covering the peak areas. Amounts calculated in dpm of both channels were plotted as separate chromatograms.

The data obtained from the RIAs were linearly plotted as the ln (mass) against the negative (—) ln $\{\%B/(100 - \%B)\}$ (where B=bound). The linearity was tested by means of a variance analysis.

RESULTS AND DISCUSSION

Fig. 1 shows a representative chromatogram (one of five experiments) of LTs and other lipoxygenase products, after [^{14}C]AA labelling of the lung tissue. The upper part of the figure represents the mass, measured by absorption at 280 nm. It is evident that substances are present with the same retention times (t_R) as LTs, indicated by C, D and B. The major compound is LTB_4, whereas LTC_4 is hardly detectable because of the strong tailing effect, caused by substances with identical chromatographical properties to some of the phospholipids, running on the front. Based on the retention time, peak 1 is tri-HETE, and peak 2 is 6-*trans*-LTB_4 + 12-*epi*,6-*trans*-LTB_4, according to Verhagen et al. [12].

The lower curve of the HPLC separation gives the plotted ^{14}C-labelled fractions. Peak 1 covers both the above-mentioned tri-HETE and a substance with the same t_R as LTC_4. Prostaglandins, however, nearly cochromatograph with LTC_4, so that a not unimportant part of peak 1 is due to the presence of cyclooxygenase products. The compound indicated by H is most probably HHT (12-OH-5,8,10-heptadecatrienoic acid). The identification of this peak was based upon the following observation: washed rat platelets were labelled with [^{14}C]AA as described before [13], aggregated with collagen, extracted as described above and applied to HPLC for further analysis. Our earlier observations on platelet aggregation indicate that the main compounds formed are, respectively, 12-HETE, HHT and TxB_2. A similar result was obtained by Luderer et al. [14]. As in our system, TxB_2 cochromatographs with LTC_4 and the retention time of 12-HETE appeared to be approx. 60 min; the peak at

26 min is likely to be HHT. In the example shown in Fig. 1, relatively large amounts of HETEs are formed.

Table III lists the recoveries (mean ± S.E.M.) of ^{3}H-labelled standards added to tissue samples. It is remarkable that the overall recoveries of HETEs are low, especially of 5-HETE. The chromatogram given at the bottom of Fig. 1

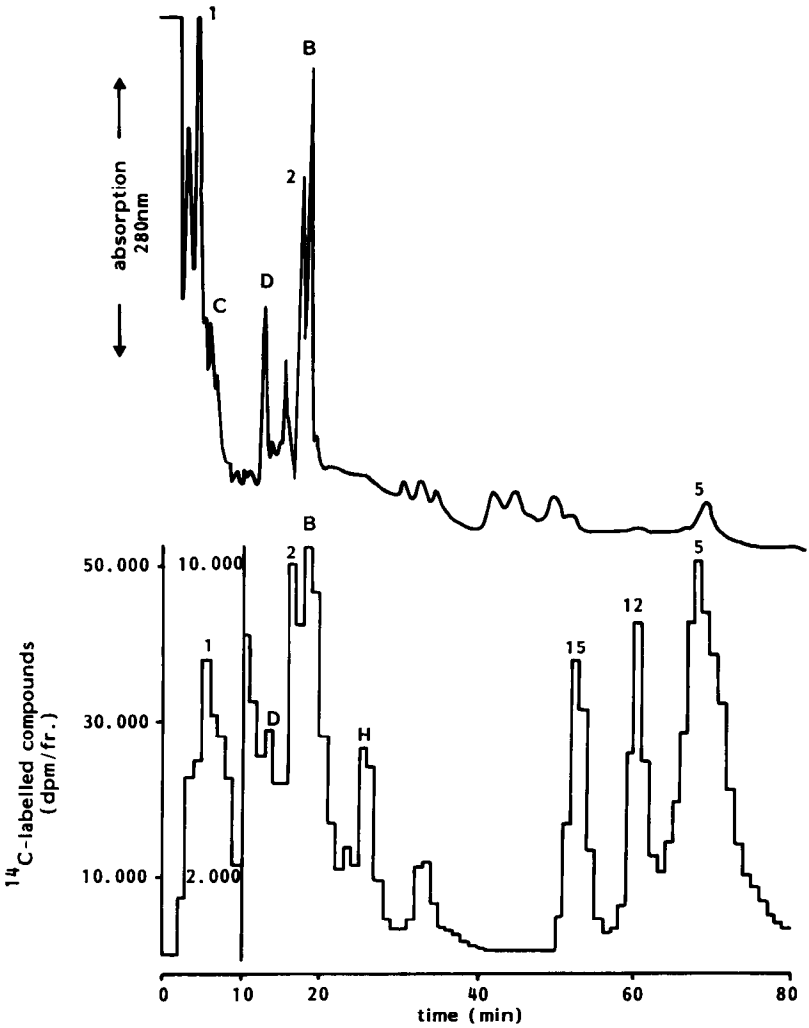

Fig. 1. Chromatograms of LTs and HETEs, synthesized by chopped human lung tissue, after [^{14}C]AA loading, in the presence of glutathione (2 m*M*) and Ca-ionophore A23187 (approx. 10 μM). The incubation medium was processed through Sep-Pak C_{18} and silica cartridges as described in Experimental and the methanol fractions after evaporation to dryness were subjected to HPLC in solvent system A. A Chrompack Nucleosil $5C_{18}$ column (250 × 4.6 mm) was used. Mobile phase: tetrahydrofuran—methanol—water—acetic acid (25:30:45:0.1) adjusted to pH 5.5 with ammonium hydroxide. Flow-rate 0.9 ml/min. The chromatogram at the bottom, representing radioactivity in fractions of one per min, is corrected for delay time between the absorption cell and the fraction collector. Peaks: C = LTC_4, D = LTD_4-like, B = LTB_4, 15 = 15-HETE, 12 = 12-HETE and 5 = 5-HETE. Based on retention times, 1 = tri-HETE, 2 = 6-*trans*-LTB_4 + 12-*epi*,6-*trans*-LTB_4.

TABLE III

RECOVERIES OF TRITIATED LTs, HETEs, AND PGs, MEASURED BY HPLC

Data were obtained after the extraction procedure as described in the methods section. Values are given as the mean ± S.E.M.

	Recovery (%)	*n*
LTC_4	59 ± 5.0	3
LTD_4-like	86 ± 1.5	3
LTB_4	70 ± 5.7	3
15-HETE	34 ± 1.1	3
12-HETE	34 ± 0.9	3
5-HETE	18 ± 0.6	3
6-Keto-$PGF_{1\alpha}$	64 ± 2.9	8
TxB_2	86 ± 2.7	8
PGF_2	44 ± 1.8	8
PGE_2	73 ± 2.9	8
PGD_2	59 ± 2.6	8

is not corrected for recoveries listed in Table III; in this case the plotted quantity of 5-HETE should be three times higher compared to the leukotrienes.

Furthermore, there is a pronounced difference in recovery of certain PGs obtained from HPLC separations as shown in Fig. 2. Fig. 2A shows the internal PG standards (^3H), fig. 2B the ^{14}C-labelled PGs formed from exogenous AA. The small peak 2 shows the immunoreactivity of 6-keto-$PGF_{1\alpha}$, and the wide peak 5 that of TxB_2. However, peak 6 (PGF-immunoreactive), peak 7 (PGE-immunoreactive) and peak 8 (PGD-immunoreactive) do not have the same t_R as the added ^3H standards. The longer the t_R, the greater the delay between ^3H-labelled and unlabelled material.

Determination of the amounts present in the sample by the different RIAs is represented in Fig. 2C. In this case, the immunoreactivities of the PGs mentioned before have the same t_R as the ^{14}C-labelled compounds. Others have noticed that a difference exists between the t_R of PGs labelled with ^3H or ^{14}C [15]. This might be due to the isotope effect. Four double bounds are labelled with ^3H whereas ^{14}C only is labelled on the 1-position. Increase of the mass plays an unimportant role, because spheric occlusion occurs at molecular weights of 2000 and higher (MW_{PG} approx. 350). It would appear that labelling with ^3H makes these substances more hydrophilic. Fig. 3 confirms the findings presented in Fig. 2. Commercially available ^3H- and ^{14}C-labelled PGE_2 and $PGF_{2\alpha}$ have a difference in retention times of approx. 5%. This could be a reason of errors in selecting the wrong fractions for RIA when ^3H-labelled standards are used as markers.

A second observation that needs further explanation is the difference that occurs in the specific activities of the PGs. This can be calculated from the data presented in Fig. 2. The pattern of ^{14}C-labelled and RIA-determined amounts is not identical. In this case, there is even a difference by a factor of 4 between the highest and the lowest specific activity (expressed as dpm/ng, covering the whole peak area). Peaks 3 and 4 in Fig. 2B are unknown. 13,14-Dihydro-15-keto-PGs run after 80 min, so that differences are not caused by these compounds.

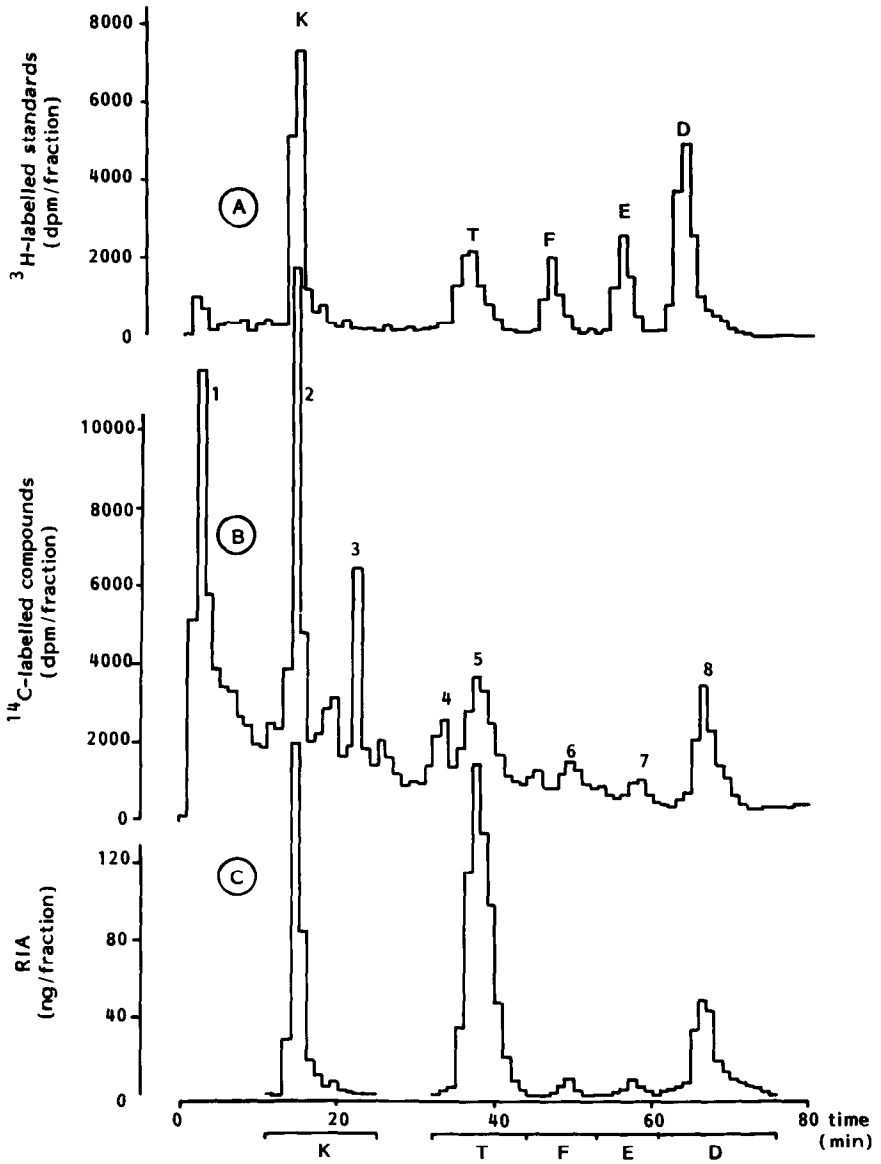

Fig. 2. Reversed-phase HPLC separation of PGs in the same sample as used in Fig. 1. A Chrompack Zorbax BP5 column was used; the solvent system contained acetonitrile—benzene—water—acetic acid (24:0.2:76:0.1); the flow-rate was 2 ml/min. One fraction per min was collected and divided as described in the text. (A) Chromatogram of [^{3}H]PG standards. Peaks: K = 6-keto-$PGF_{1\alpha}$, T = TxB_2, F = $PGF_{2\alpha}$, E = PGE_2 and D = PGD_2. (B) Chromatogram of ^{14}C-labelled compounds. Identification of peaks 1—8 is discussed in the results section. (C) Measurement of the fractions in (A) by RIA.

Fig. 4 shows the chromatograms of an experiment in which ^{3}H-labelled LTs were used as markers, without the addition of [^{14}C]AA to the tissue. Several problems arise when the LTs in the tissue are identified with the use of ^{3}H-labelled substances. This difficulty is due to the fact that in LTC_4 and LTD_4

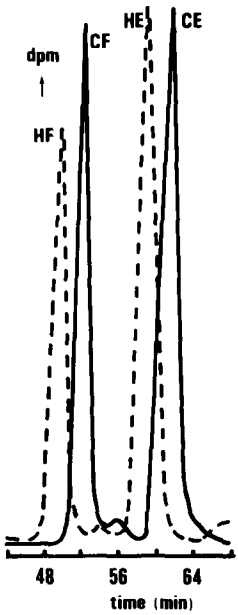

Fig. 3. Chromatogram of collected fractions (two per min) after the injection of ^{3}H- and ^{14}C-labelled $PGF_{2\alpha}$ and PGE_2. The chromatographic conditions were the same as described in Fig. 2. This chromatogram was plotted by means of a computer-programmed XY printer. Peaks: HF = [^{3}H]$PGF_{2\alpha}$, CF = [^{14}C]$PGF_{2\alpha}$, HE = [^{3}H]PGE_2, CE = [^{14}C]PGE_2.

one double bound is ^{3}H-labelled, and in LTB_4 this amount is four. The latter is comparable with the labelled PGs (see section on radiochemicals). In Fig. 4A, peaks 2 and 5 have approximately the same t_R as in Fig. 4B. The delay is only ± 2%. However, the difference is much greater between peak 6 of Fig. 4A and peak 8 of Fig. 4B. Batch 9 of the [^{3}H]LTB_4 used in the experiment as shown in Fig. 4A proved to be a racemic mixture of 6-*trans*-LTB_4 and 12-*epi*,6-*trans*-LTB_4. We thereafter injected both batch 9 and the newly prepared batch 10 directly into the HPLC system. The t_R of the first peak was the same as the one obtained in Fig. 4A after extraction procedures. The second peak (batch 10) had a delay of approx. 1 min. Compared to Fig. 4B, these two peaks were shifted 8% t_R. Peaks 3, 5 and 7+8 were collected in order to compare the biological activity of the so far unknown compound 5. The fractions were evaporated to dryness, dissolved in Krebs' buffer and added to a guinea pig lung parenchymal strip, as described before [5]. Expressed in factors of potency, the biological activities were respectively: peak 3 (LTC_4-like), 20; peak 5, 5; peak 7+8 (LTB_4-like), 1. The unknown peak 5 could be LTE_4 on the basis of its t_R [9, 16] and activity [9, 17, 18].

Finally, we determined the presence of glutamine and glycine in the hydrolysed fraction, compared to standards of LTC_4 (containing cysteine–glycine–glutamine) and LTD_4 (containing cysteine–glycine), following the method as described earlier [19]. In this fraction 5, a relatively small amount of glycine was present. We concluded from these data that this compound may be LTE_4*.

*During the preparation of the manuscript, synthetic LTE_4 was obtained. It shows the same t_R as the compound described here.

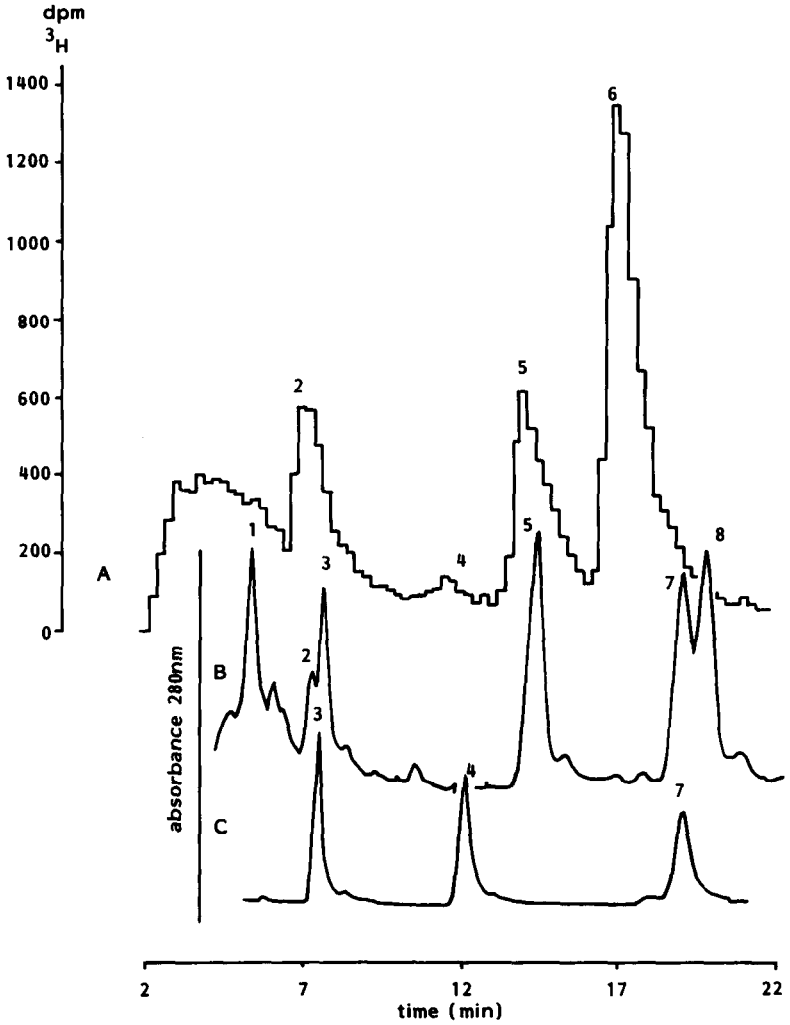

Fig. 4. Reversed-phase HPLC separation of LTC_4, LTD_4 and LTB_4. (A) ^{3}H-Labelled LTs were added to a non-labelled incubation medium of chopped human lung tissue, triggered with Ca-ionophore as described in Experimental. The numbers 2, 5 and 6 indicate the ^{3}H-labelled LTC_4, LTD_4 and LTB_4 (batch 9), respectively. (B) Measurement of the absorbance at 280 nm of the same tissue extract as mentioned in (A). (C) Chromatogram of synthetic LTC_4 (3), LTD_4 (4) and LTB_4 (7, a degradation product?), directly applied to the reversed-phase HPLC system.

When [^{3}H]LTD_4 was injected directly onto the HPLC column, the t_R was the same as peak 4 in Fig. 4A.

CONCLUSIONS

Whole human lung tissue, stimulated with Ca-ionophore A23187, produced under the conditions described, large amounts of LTB_4-like compounds and less LTC_4 and LTD_4. The major PG-like substances were immunoreactive with TxB_2, 6-keto-$PGF_{1\alpha}$ and PGD_2. The role of these different compounds has not yet been established. The formation of comparatively high amounts of TxB_2

and PGD_2 in antigen-challenged human lung tissue has been observed [20]. TxA_2 has bronchoconstrictor activity. The differences observed in the specific activities of the PGs may indicate that these substances are not formed from the same AA pool. Identification of AA metabolites could give problems when tritiated standards are used, due to the decrease of retention times. Whether recently available tritiated LTs are suitable for receptor binding studies and other specific interactions is doubtful as long as it remains uncertain that these compounds are pure LTs and not chemically degraded products.

The conversion of LTC_4 in LTD_4 by γ-glutamyltranspeptidase is inhibited by serine–borate complex [21]. Although in receptor binding studies in guinea pig lung, [^{3}H]LTD_4 was not metabolized to LTE_4 [22], this bioconversion was demonstrated in experiments with guinea pig ileum. In the presence of L-cysteine, the conversion of LTD_4 to LTE_4 was largely inhibited [23]. In future work, the effect of a 5-lipoxygenase inhibitor on the formation of PGs will be investigated, as well as the differences in AA metabolism of lung tissue obtained from asthmatics and non-asthmatics [24].

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