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Note

Comparison between ethanol and methanol for the reversed-phase high-performance liquid chromatographic analysis of leukotriene B_4 and its metabolites in cell incubation supernatants

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Polymorphonuclear leukocytes (PMNLs) convert arachidonic acid to a variety of dihydroxy metabolites, including leukotriene B_4 (LTB_4; 5*S*,12*R*-dihydroxy-6,8,10,14-*ZEEZ*-eicosatetraenoic acid). LTB_4 is formed enzymatically from leukotriene A_4, and non-enzymatic hydrolysis of LTA_4 produces the biologically inactive 6-*trans*-isomer (5*S*,12*R*-dihydroxy-6,8,10,14-*EEEZ*-eicosatetraenoic acid). LTB_4 is metabolised at the C-20 position to the 20-hydroxy metabolite which is then further oxidised to produce 20-carboxy-LTB_4. LTB_4 interacts with PMNLs to produce a variety of biological effects including chemotaxis [1], lysosomal enzyme release [2] and an increase in venular permeability [3].

We are interested in the synthesis of LTB_4 by PMNLs from arachidonic acid and its subsequent metabolism by PMNLs and a variety of other cell types including fibroblasts, endothelial cells and lymphocytes as well as the biosynthesis of deuterium-labelled eicosanoids from [$^2H_8$]arachidonic acid incubated with PMNLs in the presence of calcium ionophore A23187. The conjugated triene structure of LTB_4 and its metabolites means that high-performance liquid chromatographic (HPLC) analysis with ultraviolet detection is a suitable technique to monitor the release and metabolism of LTB_4 in many biological systems. There are many reversed-phase HPLC assays of LTB_4 and its metabolites described [4–6]. The most common organic solvents used are methanol and acetonitrile. Both of these solvents pose a health risk to chromatographers due to acute and chronic exposure.

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Health problems include blindness, enlargement of the liver and dermatological problems [7]. As skin and lung absorption of methanol is significant [8,9], concern over its toxicity encouraged us to examine the substitution of methanol with ethanol, with the aim of reducing the exposure of laboratory personnel to the toxic effects of methanol.

Methanol is the traditional solvent for most reversed-phase HPLC assays but there is no reason why ethanol cannot be substituted as a suitable alternative in many instances. However, in many countries chromatographic-grade ethanol is either not available or very expensive due to legal restrictions governing the sale of ethanol. These restrictions do not apply uniformly throughout the world and in a country such as Australia where chromatographic-grade ethanol is cheap and readily available it is often a more suitable choice than methanol as the organic component of the mobile phase for HPLC analysis and other laboratory procedures.

We have investigated the substitution of methanol with ethanol in a reversed-phase HPLC analysis for LTB_4 and associated compounds in cell incubation supernatants. We report here a solid-phase extraction procedure and gradient elution quantitation for LTB_4, its ω-oxidised metabolites and the 6-*trans*-isomer of LTB_4 comparing ethanol with methanol as the organic component of the mobile phase in these assays. This methodology has been used to purify and calculate the yield of a range of deuterium-labelled eicosanoids biosynthesised when $[^2H_8]$arachidonic acid was incubated with PMNLs isolated from peripheral blood in the presence of the lipoxygenase stimulus calcium ionophore A23187.

EXPERIMENTAL

Materials

LTB_4 (compound I), 6-*trans*-LTB_4 (compound II), 20-hydroxy-LTB_4 (compound III) and 20-carboxy-LTB_4 (compound IV) were generously provided by Merck-Frosst (Canada). Prostaglandin B_2 (PGB_2; compound V) was obtained from Sigma (Poole, U.K.). $[^2H_8]$Arachidonic acid was synthesised by reduction of 5,8,11,14-eicosatetraynoic acid with deuterium gas as reported previously [10]. Methanol was obtained from Mallinkckrodt Australia. Absolute ethanol (99.8%) was obtained from the Colonial Sugar Refining Company Australia. All other reagents used were of analytical reagent grade quality.

Chromatography

The instruments used were a Waters Model 600 multi-solvent delivery system, equipped with a Waters 712 WISP autosampler, a Waters 490 programmable multi-wavelength detector set at 270 nm and a Waters 740 data module. (Millipore/Waters, Milford, MA, U.S.A.). Extractions were performed using Sep-Pak C_{18} cartridges. Spectrophotometric analyses were performed on a Shimadzu UV 240 recording spectrophotometer (Shimadzu, Kyoto, Japan). The chromato-

graphic column used was a 5-μm Beckman Ultrasphere C_{18} (150 mm × 4.6 mm I.D.). The mobile phases consisted of either ethanol–water–acetic acid (40:60:0.02, v/v) adjusted to pH 5.6 with concentrated ammonia solution in pump A and ethanol–water–acetic acid (50:50:0.02, v/v) adjusted to pH 6.8 with concentrated ammonia solution in pump B, or methanol–water–acetic acid (58:42:0.02, v/v) adjusted to pH 6.0 with concentrated ammonia solution in pump A and methanol–water–acetic acid (71:29:0.02, v/v) adjusted to pH 7.0 with concentrated ammonia solution in pump B

Operating conditions

Gradient elution with both the ethanol and methanol mobile phase commenced with pump A supplying 100% of the mobile phase at a flow-rate of 1.0 ml/min. At 1 min a linear gradient began, which brought pump B up to 100% at 6 min and then held these conditions for 17 min. At that time a linear gradient began, which brought pump A up to 100% over a 1-min period; the flow-rate was increased to 2 ml/min. This flow-rate was maintained for a further 15 min by which time the column had re-equilibrated. The detector sensitivity was set at 1.0 a.u.f.s. and the chromatograms were attenuated at the integrator when necessary. All operations were carried out at room temperature.

Extraction procedure and standard curve preparation

Standard solutions and serial dilutions of compounds I–V were made in methanol. A standard solution containing all five compounds was used to calibrate the integrator.

Sep-Pak extraction procedure

Ethanol elution. The Sep-Pak C_{18} cartridge was prepared by flushing with 5 ml of ethanol followed by 20 ml of water. The solution to be assayed was passed through the cartridge and it was washed with 20 ml of water. Dry nitrogen was passed through the cartridge to remove residual water and the cartridge was eluted with 2 ml of ethanol. The ethanol was evaporated to dryness and the residue dissolved in 50 μl of mobile phase A (with ethanol as the organic constituent).

Methanol elution. This procedure was essentially the same as that described for ethanol except that methanol was substituted for ethanol as the organic component in the procedure.

Cell incubations

Blood (50 ml) was obtained from a healthy volunteer and the PMNLs were separated using Mono-Poly resolving medium (Flow Labs.). The cells were resuspended in 5 ml of Hanks buffered salt solution to give a final concentration of $6 \cdot 10^6$ cells per ml The cell suspension contained $>96\%$ PMNLs. Cell viability, determined by trypan blue exclusion, was more than 95%.

The cell suspension was incubated for 20 min at 37°C in the presence of 0.1 mM [$^2H_8$]arachidonic acid and 1 μM calcium ionophore A23187 (Sigma). The incubation was terminated by addition of 50 μl of 50% aqueous potassium fluoride. The resultant mixture was centrifuged to sediment the PMNLs, the cell incubation supernatant was passed through a C_{18} Sep-Pak and the extract chromatographed as described above using ethanol as the organic solvent. Fractions corresponding to the deuterium-labelled eicosanoids of interest were collected, diluted with five times the volume of water and passed through a C_{18} Sep-Pak. The material retained on the column was eluted with ethanol, a small volume of which was assayed by HPLC to determine the concentration of the compound of interest. Confirmation of peak identification and isotopic purity were performed by gas chromatography–mass spectrometry (GC–MS) as reported previously [11].

RESULTS AND DISCUSSION

Mobile phases

The gradient elution system which uses methanol in the mobile phase was developed by us and the system allows the quantitative determination of compounds I–IV in biological extracts. Fig. 1. shows the chromatogram obtained when unstimulated cell supernatant spiked with 100 ng of compounds I–IV and 50 ng of compound V was extracted and chromatographed as described above.

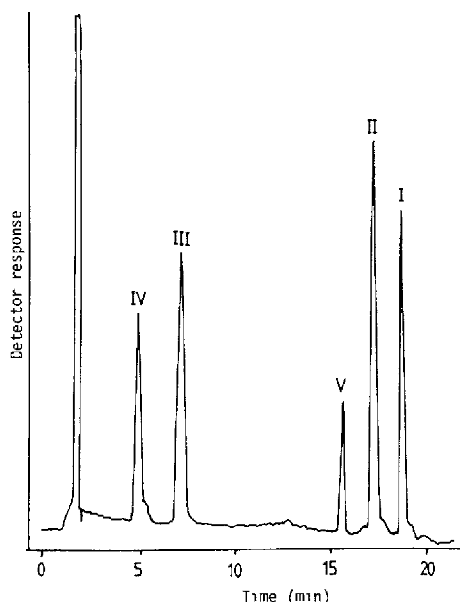

Fig. 1. Gradient elution separation of the extract from unstimulated cell supernatant spiked with 100 ng of compounds I–IV and 50 ng of compound V using methanol as the organic component of the mobile phase.

The lower pH and increased polarity of mobile phase A results in the elution of the two metabolites of LTB_4 (compounds III and IV while compounds I, II and V are retarded on the column). The latter compounds are subsequently eluted from the column by mobile phase B.

The retention times of compounds I, II and V using isocratic elution with mobile phase A is in excess of 40 min. However isocratic elution using mobile phase B results in compounds III and IV co-chromatographing and eluting almost with the solvent front. It was not possible to develop an isocratic system which could quantitate compounds I–IV.

When ethanol was substituted for methanol as the organic constituent in the mobile phase, a system which gave similar retention times for each compound was selected using the process of trial and error. A representative chromatogram analogous to that depicted in Fig. 1 is shown in Fig. 2.

Sep-Pak elution efficiency

A comparison of the efficiency of ethanol and methanol for the elution of compounds I–V from the Sep-Pak C_{18} cartridge was made by spiking Hanks buffered salt solution with each compound at 100 ng/ml and extracting five replicates using the ethanol and methanol elution procedures outlined above. Analysis of the reconstituted residues by HPLC revealed no significant difference (*t*-test) between the two extraction procedures. Ethanol elution of the Sep-Pak C_{18} cartridge was used for all subsequent studies.

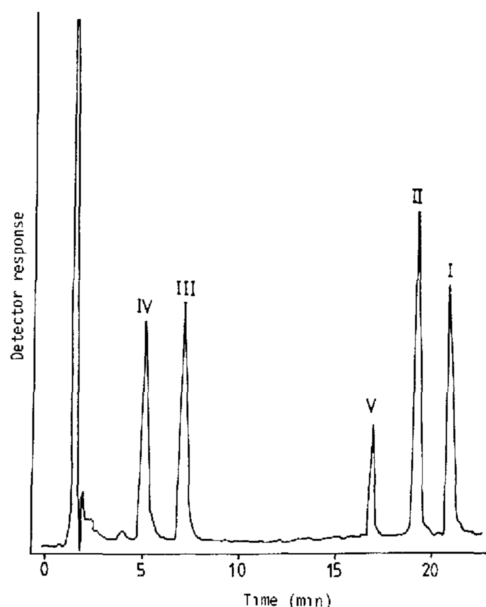

Fig 2 Gradient elution separation of the extract from unstimulated cell supernatant spiked with 100 ng of compounds I–IV and 50 ng of compound V using ethanol as the organic component of the mobile phase.

The linearity of the standard curve and the limits of detection of the HPLC assay technique for compounds I–IV were compared using ethanol and methanol as the organic component of the mobile phase.

Hanks buffered salt solution was spiked with varying amounts of compounds I–IV and a constant amount (50 ng) of compound V as the internal standard. Five replicates were extracted at 100, 60, 20 and 10 ng/ml, and the residue was reconstituted in 60 μl of mobile phase A (ethanol). A 20-μl aliquot of each sample was assayed using each of the two gradient HPLC mobile phases described above.

Table I shows the correlation coefficients of the regression line and the determination limits for compounds I–IV when the sample was assayed using the two different gradient elution systems.

As can be seen from Table I there is no difference between the linearity of the method or the limit of detection for each compound when ethanol is substituted for methanol in the mobile phase.

Cell incubation studies

The methodology described was used to purify and calculate the yield of [$^2H_8$]LTB_4, [$^2H_8$]6-*trans*-LTB_4, [$^2H_8$]20-hydroxy-LTB_4 and [$^2H_8$]20-carboxy-LTB_4 biosynthesised from [$^2H_8$]arachidonic acid by human PMNLs following stimulation with calcium ionophore A23187. The labelled eicosanoids formed have been used as internal standards in the development of the GC–MS assay for

TABLE I

CORRELATION COEFFICIENTS OF THE REGRESSION LINE AND DETECTION LIMITS FOR COMPOUNDS I–IV WHEN THE SAME SAMPLE WAS ASSAYED USING THE TWO DIFFERENT GRADIENT ELUTION SYSTEMS.

Compound	Correlation coefficient	n^a	Detection limit		n^b
			Mean (ng/ml)	C.V. (%)	
Methanol mobile phase					
I	0.9995	24	10	2.4	6
II	0.9990	23	10	2.6	6
III	0.9983	24	10	4.1	6
IV	0.9903	24	10	11.2	6
Ethanol mobile phase					
I	0.9987	24	10	3.5	6
II	0.9986	23	10	3.4	6
III	0.9991	24	10	2.9	6
IV	0.9907	24	10	10.1	6

[a] Number of determinations

[b] Number of determinations at the limit detection.

eicosanoids in complex biological matrices which will be subject of a subsequent publication.

The studies presented here show that it is possible to substitute ethanol for methanol for the procedures outlined above without adversely affecting the analytical procedure. Consequently we routinely use ethanol as the solvent of choice in our laboratory in preference to methanol where ever possible, so that our laboratory personnel are no longer at risk of acute and chronic toxicity from methanol. A further minor benefit is that the move from methanol to ethanol has cut the cost of solvents quite significantly as ethanol is not only cheaper than methanol ($A 1.90 and $A 3.03 per litre, respectively) but the same separation can be achieved using a lower proportion of organic component in the mobile phase.

The ethanol used is of similar spectroscopic purity as the HPLC-grade used for these studies. The only difference between the two solvents is that ethanol has a slightly higher UV cut-off than the methanol; however, there would be very few applications where this difference poses a problem.

Methanol is a very toxic substance which can cause necrosis of the putamen and a permanent parkinsonian-like syndrome after ingestion [12,13] and irreversible ocular toxicity [14] due to accumulation of two toxic metabolites, formaldehyde and formic acid. Since methanol is readily absorbed through the skin and lungs, chronic toxicity in the chromatography laboratory is an ever present risk. Consequently we suggest that, whenever possible, the chromatographer should endeavour to substitute ethanol for methanol in extraction procedures and chromatographic systems. Such a substitution can be justified on the grounds of safety to laboratory personnel and cost (in certain countries). There would be very few instances where there is a chromatographic rationale for the use of methanol instead of ethanol.

Indeed we contend that there is no rational reason for the continued use of methanol as the solvent of choice in HPLC analyses and that its continued use in preference to the far less toxic ethanol cannot be justified on scientific grounds. If cost (due to excise tax) and/or limited availability (due to government policy) preclude the substitution we advocate in this paper we are of the opinion that chromatographers should lobby the appropriate regulatory bodies for the removal of restrictions on the use of ethanol for laboratory purposes

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