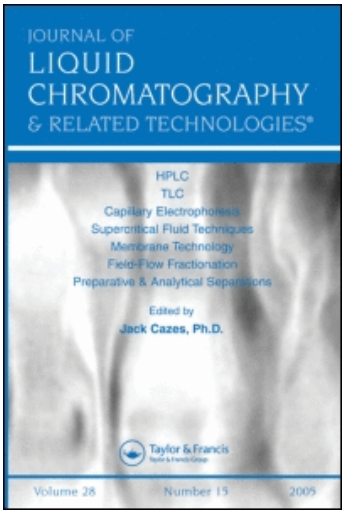

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EXTRACTION OF VARIOUS ARACHIDONIC ACID METABOLITES FROM MINIMAL AMOUNTS OF BIOLOGICAL FLUID WITH A NEW GENERATION OF MINIATURIZED SOLID PHASE EXTRACTION CARTRIDGES

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ABSTRACT

Miniaturized solid phase extraction cartridges containing phenyl silyl (PHS), ethyl silyl silica (ESS) and octadodecyl silyl silica (ODS) were compared in their applicability for efficient extraction of 5-lipoxygenase products comprising peptido-leukotrienes (p-LT's) , N-acetyl LTE_4, LTB_4, the Mono-HETE's (5/12/15), and the cyclooxgenase products TXB_2, PGE_2, PGI_2 and from minimal samples of biological fluid. Recovery rates for radiolabelled eicosanoids were standardized using 100 µl human serum or rat bile. Concurrent extraction and chemical integrity of extracted substances were monitored by RP-HPLC and RIA. Whereas ESS allowed 80%-86% recovery of prostanoids, LTB_4 and 15-HETE ODS yielded 10-20% higher recoveries. Recoveries of 12-HETE and 5-HETE were 93% and 98%, respectively, and comparable results were obtained with ODS as well as in direct recovery of p-LT's, but RP-HPLC revealed loss of radioactivity under the authentic LTC_4 peak due to the PHS-protocol. Practical applicability of the cartridges is demonstrated by bedside preparation of suction blister fluid from irritated human skin and processing rat bile continouusly obtained from the cannulated bile duct.

INTRODUCTION

Assessment of eicosanoids as potent mediators of inflammation and cell communication has become an important scope of scientific work. Usual experiments in which arachidonic acid (AA) metabolites are measured have the following characteristics: 1) they are done in short-term cell or tissue cultures 2) they are done with highly concentrated and homogeneous cell populations 3) the cultures are usually supplemented with AA and 4) forced to synthesize vast amounts of eicosanoids with calcium ionophore 5) which are extracted from buffer solutions or other protein-depleted fluids. In contrast, extraction of eicosanoids from biological fluids still represents a methodological challenge because 1) the samples contain high amounts of proteins and 2) metabolizing enzymes 3) the volume of samples is, particularly in laboratory animals, limited 4) the amounts of eicosanoids range far below the amounts obtained in cell experiments. Recently, miniaturized versions of solid phase extraction cartridges containing 100 mg sorbent only (PHS, ESS AMPREP and ODS SepPak LIGHT) became available, which we regarded as valuable tools to solve our problems with limited amounts of biological fluids (human suction blister fluid and rat bile). Protocols for extraction of p-LT's with PHS-AMPREP and for prostanoids and LTB_4 with ESS- AMPREP are given by the producer. An overall recovery for p-LT's of 93% and over 90% for prostanoids and LTB_4, respectively are claimed. The producer of SepPak Light ODS refers to a large body of literature, which gives various and differing recovery rates for several AA metabolites obtained with the larger cartridges (for review see 1 and 2).

MATERIAL AND METHODS

[^{3}H]-LTB_4 (spec. act. 190.0 Ci/mmol), [^{3}H]-LTC_4 (spec. act. 38.4 Ci/mmol), [^{3}H]-LTD_4 (spec. act. 38.4 Ci/mmol), [^{3}H]-LTE_4 (spec. act. 38.4 Ci/mmol), [^{3}H]-TXB_2 (spec. act. 114.0 Ci/mmol), [^{3}H]-PGE_2 (spec. act. 185.0 Ci/mmol) and [^{3}H]-PGI_2 methyl ester (spec. act. 16.0 Ci/mmol) were purchased from NEN, Dupont, Dreieich, FRG. The tritiated Mono-HETE's 5,12, and 15 (spec. act. 191, 172 and 213 Ci/mmol, respectively) were obtained from Amersham-Buchler, Braunschweig, FRG as well as the AMPREP PHS and ESS minicolumns (100 mg sorbent). [^{3}H]-Nac-LTE_4 was a generous

gift from Dr. D. Keppler, DKFZ, Heidelberg. SepPak LIGHT ODS cartridges were from Waters, Eschborn, FRG. Scintillation cocktail (Ready Safe), polyethylene minivials and the LS 1701 beta-counter from Beckmann Instruments, Munich, FRG, were used. Methyl formate (Mef) in spectrophotometric grade was purchased from Aldrich, Steinheim, FRG. All other reagents were of analytical grade or HPLC quality (Merck, Darmstadt or Roth, Karlsruhe, FRG). 10% ethanol/8% HCl as requested in the protocol for PHS was prepared by adding 25 ml 32% HCl and 10 ml ethanol (EtOH) to 65 ml water. P-LT's were further assessed after RP-HPLC using a RIA kit from Amersham-Buchler, Braunschweig, FRG.

Preparation of Samples

1 ml serum was obtained from human donor blood and doped with 10 µl (0.25 µCi) of the respective tracer. The final column loads with tracer were 0.26 pmol for p-LT's, 0.12-0.15 pmol for the HETE's, 0.6 and 0.9 pmol for PGE_2/PGI_2 and TXB_2, respectively. These concentrations are rather low but still within the detection limits of commercial RIA kits. The doped serum was allowed to stand for 7 minutes on ice and then acidified to pH 3 by adding 130 µl 1N HCl (PHS and ESS) or 30 µl 85% formic acid (ODS) and 100 µl samples were passed through the columns. Because preceding experiments had shown some seroconversion of LTC_4 (up to 30%) 4 mM acivicin and 4 mM D-penicillamin (3,4) were added to sera at least two hours prior to addition of tracer. These concentrations proved to be sufficient to prevent seroconversion under our experimental conditions. 50 µM indomethacin was used accordingly for prostanoid extraction experiments. In order to exclude enzymatic conversion and possible masking of recovery rates a solution was made from phosphate buffered saline (pH 7.4) and 0.6 mg/ml essential fatty acid free human serum albumin (HSA, fraction V, SIGMA, Munich, FRG). This solution was acidified with 10 µl 85% formic acid or 75 µl 1 N HCl, respectively.

Data Acquisition for direct Recovery Determination

Immediately before extraction four 100 µl portions were separately pipetted into 5 ml scintillation liquid serving as total counts (TC) samples. Accordingly, the extraction procedures were

run in quadruplicates to allow determination of average counts, standard deviation (SD) and coefficient of variance (CV) for every batch of elution experiments. The final eluates were collected in a minivial, dried under argon and resuspended in the initial volume of (acidified) serum or other fluids to obtain a quench identical to the total count (TC) samples. 5 ml scintillation liquid was added and the mixture was shaken vigorously. The scintillation cocktail was allowed to equilibrate overnight and radioactivity was counted in cpm for one minute.

Protocols

The AMPREP columns were conditioned with 2 ml methanol (MeOH) followed by 2 ml distilled water, SepPAK LIGHT with 2 ml MeOH and 2 ml 0.025% formic acid (pH 3). Further elution steps:

PH AMPREP for P-LT's

step 1 2 ml 10%EtOH/8% HCl
step 2 2 ml water
step 3 2 ml hexane
step 4 2 ml methyl formate (Mef)
step 5 1 ml MeOH into the same vial as for step 4

ESS AMPREP for Prostanoids, LT B_4 and Mono-HETE's

step 1 2 ml water
step 2 2 ml 10% EtOH
step 3 2 ml hexane
step 4 2 ml Mef
step 5 1 ml MeOH into the same vial as for step 4

ODS SepPak LIGHT

step 1 2 ml 10% EtOH
step 2 5 ml 0.025% formic acid
step 3 2 ml hexane
step 4 2 ml MeOH

Biological Fluids

Under deep barbital anaesthesia the bile duct of male Wistar rats was cannulated with a polyethylene tube and bile continuously collected. Every 15 minutes fractions giving (90-120 µl) were withdrawn for the ODS protocol. Human suction blister fluid was obtained from volunteers as described elsewhere (5). The blisters were punctured and the fluid aspirated into tuberculin plastic syringes, the volumes ranging from 20 µl to 200 µl. The samples were acidified with formic acid and immediately extracted with SepPak LIGHT at the bed side.

Reversed-Phase High Pressure Liquid Chromatography (RP-HPLC)

The final eluates were dried under argon, resuspended in 10% MeOH and subjected to RP-HPLC, which was performed with a Waters (Eschborn, FRG) model 5/o HPLC pump, a U6K injector and a Lambda Max LC spectrophotometer with a variable wave length UV-detector (280 nm for prostanoids and LT's and 237 nm for mono-HETE's). Eicosanoids were purified on a reversed phase (250x4 mm) column packed with Hypersil ODS 5 µm guarded by a Hypersil ODS 15x4 mm, 5 µm precolumn (all LATEK, Eppelheim, FRG). The solvent system MeOH:water:acetic acid (733:296:1) was adjusted to pH 6.1 with 25% NH4OH and Na-EDTA (437 mg/ml). The mobile phase flow rate was 0.9 ml/minute. Standard chromatograms were obtained with authentic eicosanoids (PAESEL, Frankfurt, FRG). The RP-HPLC recovery rates were determined by injection of radiolabelled standards. Fractions were collected every 6 seconds and radioactivity monitored.

RESULTS

Recovery of P-LT's before RP-HPLC

For p-LT's no major differences were found in direct recovery determination from serum (tab. 1) or PBS/HSA (not shown). The percentages are the mean of 4 determinations showing standard deviation and coefficient of variance in brackets. With HCl concentrations lower than specified in the PHS AMPREP protocol, the recoveries did not exceed 45 %. Extraction of p-LT's from rat bile was only done with ODS and gave lower yields (tab. 1).

TABLE 1

Directly determined apparent Recovery Rates for P-LT's from 100 µl Human Serum and Rat Bile (only ODS), respectively.

	PHS AMPREP serum	ODS (C18) SEPPAK serum	LIGHT rat bile
LTC_4	73% ± 7 (9.6)	73% ± 3 (3.8)	78% ± 2 (2.9)
LTD_4	93% ± 7 (7.2)	98% ± 3 (3.4)	81% ± 4 (4.9)
LTE_4	87% ± 1 (1.3)	95% ± 7 (7.4)	77% ± 5 (6.5)
Nac-LTE_4	not done	not done	82% ± 1 (1.3)

TABLE 2

Recovery Rates of radiolabelled Prostanoids, LTB_4 and Mono-HETE's.

	ESS AMPREP		ODS SepPAK LIGHT	
TXB_2	80% ± 1	(1.7)	86% ± 5	(6.2)
PGE_2	83% ± 2	(2.5)	100% ± 4	(3.6)
PGI_2	86% ± 3	(2.9)	99% ± 5	(4.6)
LT B_4	81% ± 4	(4.6)	91% ± 8	(5.0)
15-HETE	81% ± 6	(7.8)	100% ± 5	(5.0)
12-HETE	93% ± 3	(2.6)	92% ± 7	(7.0)
5-HETE	98% ± 2	(2.2)	94% ± 7	(7.0)

Immediately after acidification flocculent precipitation of bile acids occurred. The bile was cleared by centrifugation at 12000 G for 2 minutes without significant loss of radioactivity into the pellet. Extraction of prostanoids, LTB_4 and 15-HETE with ODS gave significantly higher yields than with ESS, whereas results with 5- and 12-HETE were comparable (tab. 2).

RP-HPLC

Typical retention times in standard chromatograms were 4.6 minutes for PGE_2, 5.5 minutes for LTC_4, 6.9 minutes for Nac-LTE_4, 9.0 minutes for LTD_4, 9.8 minutes for LTB_4, 10.4 minutes for LTE_4, 24.2 minutes for 15-HETE, 28.0 minutes for 12-HETE and 31.8 minutes for 5-HETE. Determination of radioactivity in HPLC-fractions of tritiated standards gave the following recovery rates: PGE_2: 81%, LTC_4: 97%, Nac-LTE_4: 95%, LTD_4: 90%, LTB_4: 92%, LTE_4: 87%, 15-HETE: 98%, 12-HETE: 93% and 5-HETE: 95%. Surprisingly, the radioactivity under the authentic LTC_4 peak from PHS-extracted samples was markedly reduced in comparison to ODS (fig. 1), although the directly determined recovery rates seemed to be almost identical (comp. tab. 1). Flushing the RP-HPLC column with pure MeOH released radioactive material retained under isocratic conditions. The losses accounted for approximately 20-40% of injected tracer and were reproducibly observed with different serum batches and with PBS/HSA. Examples for concurrent extraction of various AA metabolites with ODS are demonstrated in figures 2 and 3.

Practical application

Excretion of p-LT's and Nac-LTE_4 in rat bile reached a basal level 90 minutes after beginning of abdominal surgery, (fig. 4) with Nac-LTE_4 being the predominant metabolite. P-LT's were also found in human suction blister fluid, ranging from 73 pg/ml to 5.1 ng/ml (not shown).

DISCUSSION

We tested three different sorbent materials in minicolumns for their ability to extract various AA metabolites from minimal

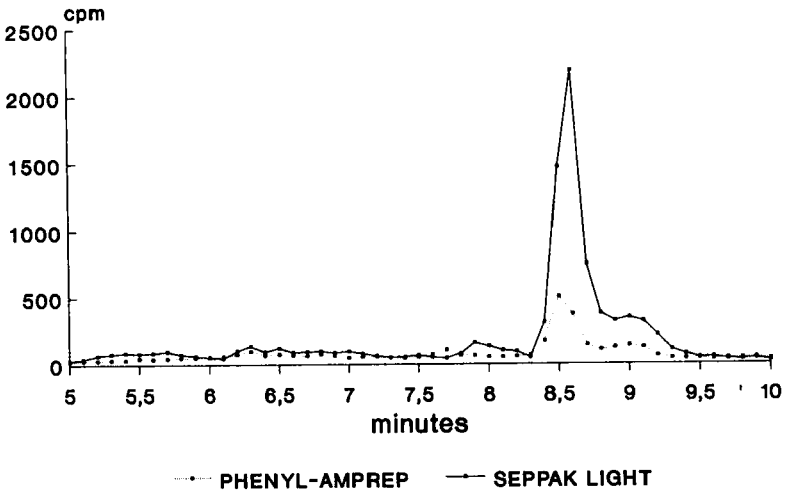

FIGURE 1: Radiochromatogram of tritiated leukotriene C_4 extracted from 100 µl human serum using PHS or ODS. Although identical volumes from the same doped serum batch were extracted, the yield with ODS is almost four times that of PHS. Identical chromatograms were obtained with extraction from PBS/albumin.

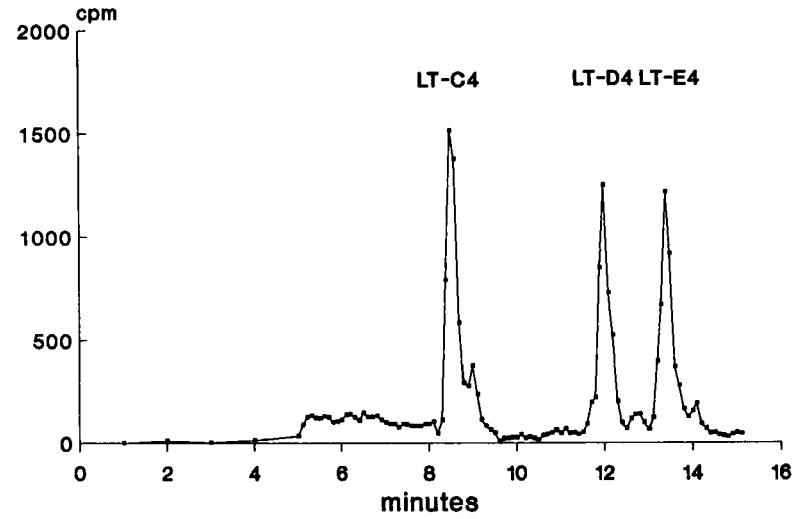

FIGURE 2: Concurrent simultaneous extraction of tritiated p-LT's. The tracers were dissolved in PBS containing 0.6 mg/ml essential fatty acid free HSA and extracted with the ODS minicolumn. Similar chromatograms were obtained with serum containing acivicin and D-penicillamine.

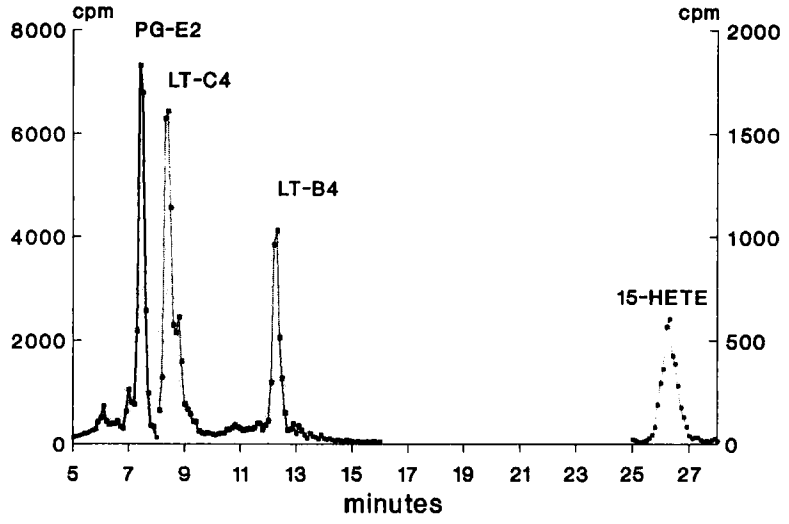

FIGURE 3: Concurrent simultaneous extraction of tritiated PGE_2, LTC_4, LTB_4 and 15-HETE extracted from 100 µl human serum inhibited with acivicin and D-penicillamine. Note, that the radioactive PGE_2 peak refers to the left y-axis.

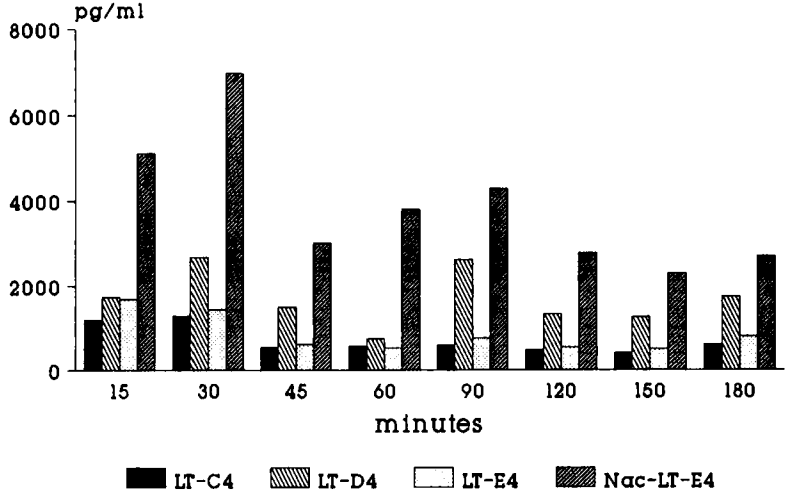

FIGURE 4: Time course profile of biliary p - LT excretion during abdominal surgery in the rat. 30 minutes after opening of the abdominal cave and cannulation of the bile duct a secretory maximum due to surgical trauma is evident. After another two hours an equilibrium is reached which would allow measurement of p-LT exretion upon different stimuli.

amounts of biological fluids. Because we felt that recovery studies done with labelled eicosanoids extracted from pure buffer may not be representative, serum was chosen as proteinaceous standard fluid. In fact, recovery rates are comparably lower with proteinaceous fluids (6, 7). The problem of seroconversion of LTC_4 to LTD_4, which would have interfered with the correct recovery determination for LTC_4 was overcome with appropriate enzyme inhibitors and keeping samples on ice. The application of acivicin and D-penicillamin to human serum 2 hours before extraction does not represent real experimental conditions, but allowed us, to elaborate real recovery rates for LTC4 as confirmed by another model fluid, PBS/HSA which did not contain LTC_4 converting enzymes. This solution was prepared, because there is growing evidence that albumin interacts with AA and some of its oxygenated metabolites (8, 9). In contrast, bile represents a complex mixture of water soluble and non soluble substances in micellar arrangement with a comparably low protein content and therefore was tested separately in our extraction experiments with ODS. Concerning the extraction of p-LT's the PHS protocol turned out to produce a pseudorecovery as determined directly, which was unmasked by RP-HPLC revealing losses of radioactivity under authentic LTC_4. Most probably the striking amounts of HCl as recommended in the PHS protocol (and necessary to obtain considerable elution of radioactive material) leads to a physico-chemical modification of p-LT's altering at least their retention behavior in RP-HPLC. In our opinion this protocol and the sorbent is not advisable in extraction with subsequent RP-HPLC analysis. In contrast, the ESS procedure proved to be a valuable tool in extraction of the other 7 AA metabolites. It was impossible to extract p-LT's with ESS and vice versa, PHS was not suitable for extraction of prostanoids and the HETE's. Only with ODS good to excellent recovery rates were obtained for all 11 metabolites with the protocol whe had developed for this sorbent type. Especially for the mono-HETE's the yield was remarkably high in comparison to other studies reporting 45% for 15-HETE (1, 2) or 80% for 12-HETE (10).

In view of the fact, that p-LT plasma levels are invariably low, it was not intended in this study to suggest, that extraction of 100 µl serum would yield enough assayable material, rather than to use serum as standardizing proteinaceous fluid. However, we could demonstrate, that 100 µl suction blister fluid, which is aspirated from subepidermal blisters filled with a serum filtrate contains enough assayable AA metabolites in the blister compartment. P-

LT's, wherever formed in the organism (of probably all mammals) are biologically inactivated by hepatocytic N-acetyl transferase and excreted via the bile. Therefore it does not seem to be too surprising to find sufficient amounts of p-LT's already in 100 µl bile. As to be expected, Nac-LTE$_4$ was the predominant metabolite in this biological fluid.

Preliminary data suggest that the recovery rates obtained with SepPak LIGHT are constant up to 250 µl sample volume. Volumes exceeding 250 µl should be applied at least twice or the greater cartridges used. However, the miniaturized ODS cartridges meet the criteria for rapid, efficient and concurrent extraction of a multitude of AA metabolites and thus represent a valuable help in analyzing minimal amounts of biological fluids.

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