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Note

Rapid extraction of arachidonic acid metabolites utilizing octadecyl reversed-phase columns

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Prostaglandins (PGs), thromboxanes (TXs) and other arachidonic acid metabolites are usually extracted from acidified plasma, urine or tissue samples with organic solvents such as diethyl ether, ethyl acetate or chloroform [1]. The solvents are then evaporated, the residues resuspended and oftentimes purified further with silicic acid column chromatography prior to high-performance liquid chromatography (HPLC), radioimmunoassay (RIA), or gas chromatography—mass spectrometry (GC—MS). If it is necessary to remove neutral lipids, the aqueous samples must first be extracted with petroleum ether prior to acidification and further extraction. Such procedures employing multiple solvent extraction steps and column chromatography are time consuming and frequently require the evaporation of relatively large volumes of organic solvent.

We have developed a sample preparation procedure which eliminates solvent extraction by utilizing the Baker 10 Extraction System (J.T. Baker, Phillipsburg, NJ, U.S.A.). This system was originally developed for the extraction of drugs from plasma samples and consists of a specially designed vacuum manifold capable of processing up to ten columns simultaneously (see Fig. 1). Commercially available, disposable columns are prepacked with octadecyl (C_{18}) silane bonded to silica gel. Acidified samples (plasma, urine, cell suspensions) containing prostaglandins and related compounds can be applied directly to the column without prior solvent extraction and selectively eluted. This technique is more rapid than the conventional silicic acid column chromatography utilized in separating out prostaglandins. The samples pass through the columns with the assistance of a vacuum and compounds can be eluted with small volumes (< 500 μl) of solvent. In addition to preparing samples for RIA and

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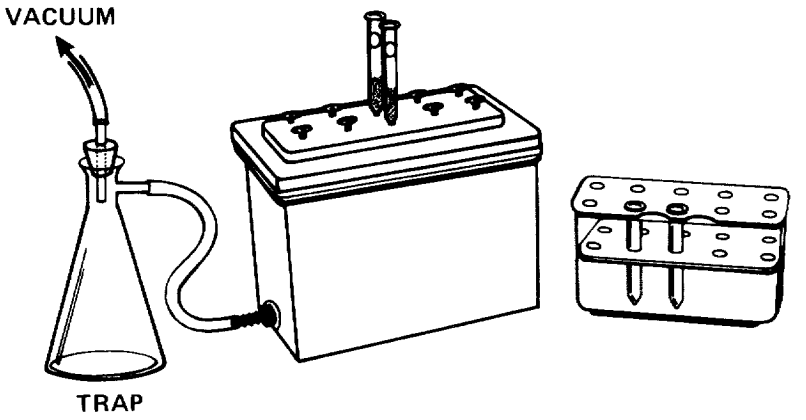

Fig. 1. Baker 10 Extraction System. Up to ten disposable extraction columns can be processed simultaneously. Solvents and samples pass through the columns by application of a vacuum. Sample eluents are collected in tubes positioned in a removable rack placed inside the manifold.

HPLC, we have also utilized this technique in lieu of organic solvent extraction to remove substances from aqueous HPLC mobile phases prior to derivatization for GC–MS. Finally, an HPLC method utilizing radial compression columns is also described for the separation of human granulocyte lipoxygenase products of arachidonic acid metabolism.

MATERIALS AND METHODS

[^{14}C]Arachidonic acid and tritiated standards of arachidonic acid, PGB_2, PGD_2, PGE_1, PGE_2, $PGF_{2\alpha}$, TXB_2, 5-hydroxyeicosatetraenoic acid (5-HETE), 12-HETE, Leukotriene C_4 (LTC_4), Aquasol and radioimmunoassay kits for TXB_2 were purchased from New England Nuclear (Boston, MA, U.S.A.). [^{3}H]6-Keto $PGF_{1\alpha}$ was purchased from Amersham (Arlington Heights, IL, U.S.A.). [^{3}H]LTB_4 was biosynthesized from human granulocytes and purified by HPLC as described. Unlabelled arachidonic acid (purity > 99%), and nordihydroguaiaretic acid were obtained from Sigma (St. Louis, MO, U.S.A.). Diazomethane was prepared from N-methyl-N-nitroso-N-nitroguanidine (Sigma). Ionophore A23187 was purchased from Calbiochem (La Jolla, CA, U.S.A.). Ficoll–Paque was purchased from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). HPLC solvents were purchased from Fisher (Pittsburgh, PA, U.S.A.) and J.T. Baker and passed through a Millipore filter (0.5 μm) prior to use. Indomethacin was a gift from Merck Sharp and Dohme (West Point, PA, U.S.A.). LTB_4 standard was synthesized in the laboratory of E.J. Corey and was a generous gift from Dr. Susan Levinson (Ortho Pharmaceuticals, Rahway, NJ, U.S.A.). 5-HETE was also a gift from Dr. Levinson.

Preparation of columns and extraction procedure

Octadecyl (C_{18}) extraction columns (3 ml) were placed in the luer fittings of the Baker-10 Extraction System manifold cover (see Fig. 1). A vacuum (380–500 mm Hg) was applied and the column washed with methanol (2–3 ml)

followed by 2–3 ml of phosphate buffered saline (pH 3), and the vacuum turned off so as not to allow the sorbent to dry. The methanol wash is necessary to condition the packing material to allow aqueous samples to pass through it. The conditioning flush with buffer is necessary to prepare the packing material surface for adsorption. A pH 3 buffer is used for the conditioning step since aqueous samples were applied to the column at this pH. The volumes delivered to the column in these conditioning steps are not critical and were added from plastic squeeze bottles. Plasma, buffer, or urine samples were acidified (pH 3) with either 1 *N* hydrochloric acid or 2 *N* formic acid. The plasma (1 ml) and buffer (1 ml) samples were applied directly to the column. Small urine volumes (10 ml) could be applied directly, but when larger volumes (> 50 ml) were processed, the acidified urine was centrifuged (1000 *g*) before application to the column since urine sediment has a tendency to clog the column. Large volumes of urine (400 ml) were processed using a 6-ml octadecyl (C_{18}) column fitted with a reservoir which attaches to the top of the column to accommodate the larger sample size.

High-performance liquid chromatography

Chromatography was performed with a Waters Assoc. HPLC system (U6K injector, two 6000A pumps, data module, system controller, Z-module radial compression system). Radial-Pak μBondapak C_{18} cartridges (10 cm × 8 mm, 10 μm) or fatty acid analysis stainless-steel columns (30 cm × 3.9 mm) from Waters were used. Linear solvent gradients from 45% acetonitrile (with 0.1% glacial acetic acid) to 65% acetonitrile (with 0.1% glacial acetic acid) were delivered at a flow-rate of 3 ml/min for the separation of lipoxygenase products. Absorbance was monitored with a Waters 440 detector (280 nm) and a Schoeffel (SF 770) variable-wavelength detector (235 nm) connected in series. Fractions were collected with a Gilson (B-200) automatic fraction collector. A guard column packed with μBondapak C_{18} was also employed.

Preparation of platelets and granulocytes

Blood was obtained from normal volunteers who had not ingested aspirin or nonsteroidal anti-inflammatory agents for at least two weeks. Washed platelet suspensions were prepared as previously described [2, 3]. To prepare granulocytes, whole blood was drawn into a plastic syringe containing heparin (500 U in 0.5 ml) in 6% clinical dextran (3.13 ml per 15 ml blood). After gentle mixing the cells were allowed to sediment for 90 min at room temperature. The plasma was removed from the sedimented red cells and centrifuged (180 *g*) for 8 min. The cell pellet was resuspended and washed twice in Tris-buffered saline (pH 7.4; 0.15 *M* sodium chloride) containing heparin (5 U/ml). The washed cells were then layered onto a Ficoll–Paque gradient (sp.gr. = 1.078) and centrifuged at 20°C according to the method of Boyum [4]. The lower granulocyte layer was then resuspended in 5 ml Tris-buffered isotonic ammonium chloride (pH 7.2) and incubated for 4 min (37°C) to lyse contaminating red cells [5]. After centrifugation (180 *g*) for 4 min the cells were washed again in ammonium chloride, and finally resuspended in phosphate-buffered saline (PBS). The PBS was prepared as previously described [6]. The cell suspension contained > 98% granulocytes which were > 95% viable as assessed by trypton-blue dye exclusion.

Platelet and granulocyte incubations

Washed platelet suspensions (0.4 ml; 10^9 cells/ml) were stirred in a platelet aggregometer (1100 rpm) at 37°C. Following the addition of arachidonic acid (1 μg) the platelet incubation was continued for 15 min. Granulocyte suspensions (0.5 ml; 10^6 cells/ml) were stirred in a constant temperature circulating water bath at 37°C. Ionophore A23187 was added (final concentration, 20 μM) and 5 min later arachidonic acid (final concentration, 75 μM) was added and the incubation continued for 15 min. Preliminary studies in our laboratory have indicated that these reaction conditions result in maximal production of LTB_4. The incubation was terminated by the addition of methanol (1 ml) and 2 *N* formic acid (to adjust the pH to 3—3.5). After centrifugation the supernatant was applied to the octadecyl column as described and eluted sequentially with water (2 ml), 25% methanol (2 ml) and 100% methanol (2 ml). The methanol fraction was taken to dryness (in vacuo) and resuspended in methanol (100 μl) prior to HPLC.

RESULTS AND DISCUSSION

Table I lists the recoveries (mean ± 1 S.D.) of radiolabelled standards extracted from buffer. The recoveries range from 73 to 98% with an average recovery of 84%. These values represent the percent of radioactivity recovered in the methanol fraction after the prepared columns were eluted sequentially with water (2 ml), 25% methanol (2 ml) and 100% methanol (2 ml). The usual procedure of solvent extraction followed by silicic acid column chromatography commonly results in recoveries of less than 70% [1]. The majority of the unrecovered radioactivity was found in the eluate obtained when the

TABLE I

RECOVERIES OF STANDARDS EXTRACTED FROM BUFFER USING OCTADECYL (C_{18}) 3-ml COLUMNS

Columns were prepared as described in Methods and eluted sequentially with 100% water (2 ml) 25% methanol—water (2 ml), and 100% methanol (2 ml).

Compound	Recovery (%)* (Mean ± S.D., n = 4)
Arachidonic acid	79 ± 6
PGB_2	87 ± 2
PGD_2	88 ± 2
PGE_2	82 ± 2
$PGF_{2\alpha}$	88 ± 4
6-Keto $PGF_{1\alpha}$	73 ± 4
TXB_2	79 ± 2
LTB_4	98 ± 2
5-HETE	77 ± 10
12-HETE	88 ± 9

*Recovery in the 100% methanol fraction.

samples were applied to the column. Lesser amounts of radioactivity were recovered in the fractions resulting from elution with water and 25% methanol. Washing the column with water and 25% methanol was found to be necessary when preparing incubations from granulocytes or platelets for HPLC. These clean-up steps removed interfering UV-absorbing peaks and proteinaceous material. Fig. 2 shows a representative chromatogram obtained from the methanol fraction of a granulocyte incubation carried out with [^{14}C]-arachidonic acid. [^{3}H]PGB_2 (400 ng, 50,000 cpm) was added as an internal standard following the incubation to calculate recoveries.

The identification of the peak designated as LTB_4 in Fig. 2 was based upon the following observations: (1) As depicted in Fig. 2, this peak incorporated a ^{14}C-label when incubations were performed with [^{14}C]arachidonic acid. (2) This peak was inhibited when granulocyte incubations were carried out in the presence of the lipoxygenase inhibitor nordihydroguaiaretic acid (30 μM) [7] but not with indomethacin (10^{-4} M). (3) When this peak was collected, separated from the aqueous mobile phase [using 3-ml octadecyl (C_{18}) extraction column as before] and derivatized with ethereal diazomethane, the resultant product after HPLC purification, showed a typical triene UV absorption pattern with peaks at 259, 270, and 281 nm. This is in agreement with spectral data for the methyl ester of LTB_4 as reported by Samuelsson et al. [8]. (4) Finally, the retention time of this peak corresponded with that of authentic, chemically synthesized, LTB_4. Moreover, when this peak was collected and mixed with authentic LTB_4 and rechromatographed, the mixture

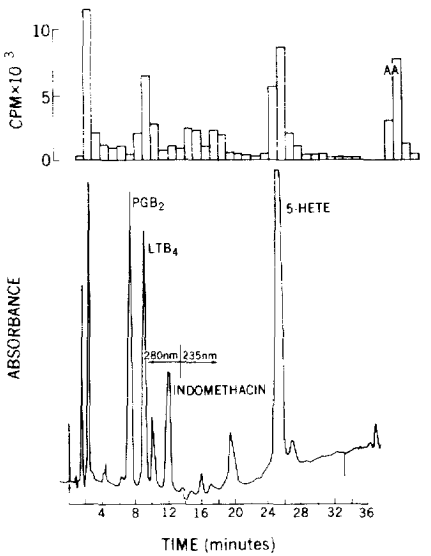

Fig. 2. HPLC separation of human granulocyte lipoxygenase products. Granulocyte incubations were carried out in the presence of indomethacin (10^{-4} M) and [^{14}C]arachidonic acid. The incubation medium was processed through an octadecyl (C_{18}) silane column as described in Methods and the methanol fractions subjected to HPLC. PGB_2 was added as an internal standard. A Waters radial compression μBondapak C_{18} cartridge (10 cm × 8 mm, 10 μm) was used. A linear solvent gradient from 45% to 65% acetonitrile in water (with 0.1% glacial acetic acid) was delivered at a flow-rate of 3 ml/min.

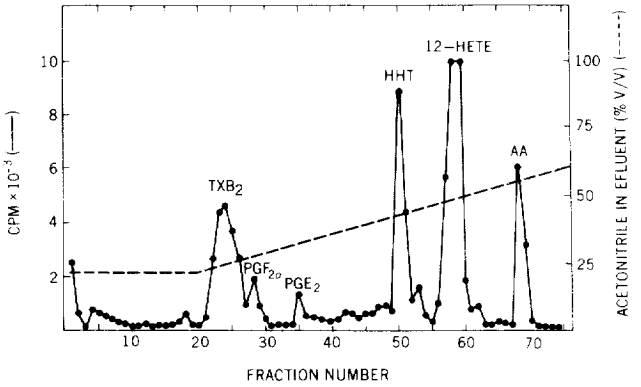

Fig. 3. HPLC separation of reaction products from human platelets incubated with [^{14}C]-arachidonic acid. The incubation medium was processed through an octadecyl (C_{18}) silane column as described in Methods and the methanol fraction subjected to HPLC. A Fatty Acid Analysis stainless-steel column (30 cm × 3.9 mm) was used. The initial eluent was 23% acetonitrile (with 0.1% glacial acetic acid), and after 20 min the acetonitrile was increased over a linear gradient as shown. The flow-rate was 2 ml/min. Fractions were collected and radioactivity determined by liquid scintillation counting.

eluted as a single component. The identity of 5-HETE was similarly established by means of incorporation of [^{14}C]arachidonic acid, inhibition with nordihydroguaiaretic acid, and co-elution with chemically synthesized 5-HETE and purchased [^{3}H]5-HETE.

Fig. 3 shows a representative chromatogram obtained when washed human platelets were incubated with [^{14}C]arachidonic acid and illustrates the utility of this extraction technique in experiments utilizing platelets. The incubation medium was processed exactly as outlined for the granulocytes. The identification of the peaks was as previously described [2].

Table II lists the recoveries obtained from plasma samples spiked with radiolabelled standards. This technique was developed for processing plasma samples

TABLE II

RECOVERIES OF STANDARDS FROM HUMAN PLASMA USING OCTADECYL (C_{18}) 3-ml COLUMNS

Columns were prepared as described in Methods and eluted sequentially with water (2 ml), benzene (2 ml), and ethyl acetate (2 ml).

Compound	Recovery (%)* (Mean ± S.D., n = 4)		
	Water	Benzene	Ethyl acetate
Arachidonic acid	5.0 ± 2.6	35.1 ± 9.9	6.8 ± 1.8
PGE_1	0.9 ± 0.1	0.3 ± 0.1	79.9 ± 4.2
PGE_2	2.8 ± 0.2	1.3 ± 0.2	74.4 ± 2.9
$PGF_{2\alpha}$	1.5 ± 0.1	0.9 ± 0.1	74.8 ± 2.1
TXB_2	2.2 ± 0.2	1.1 ± 0.1	79.1 ± 5.0

*Counts used in calculating recoveries were corrected for quenching.

for RIA. As shown, when acidified plasma (pH 3) was applied to the column and eluted with water, the majority of the radioactivity remained on column. This aqueous wash step, however, removed water-soluble components in unextracted plasma that could interfere with the subsequent RIA. The columns were then eluted with benzene, and as demonstrated using arachidonic acid as a marker, this step removes fatty acids while allowing the prostaglandins and thromboxanes to remain on column thus avoiding the double solvent extraction described in previous methods [1]. Finally, Table II demonstrates that the prostaglandins of interest can then be eluted in high yield with ethyl acetate. The ethyl acetate can then be volatilized easily under a stream of nitrogen. With the specificity of antibodies that are now available, the fact that the various prostaglandins were present in a mixture has not presented a problem. If desired, it would probably be possible to devise a more selective solvent system to separate the various prostaglandins. As can be seen in Table II, only about 42% of the arachidonic acid can be accounted for in the benzene and ethyl acetate fractions combined. The majority of the remaining arachidonic acid was eluted when the plasma sample was applied to the column. This probably results from the fact that a significant portion of the arachidonic acid is bound to protein that is not retained by the column, since this loss was not observed when arachidonic acid was applied in a protein-free buffer (Table I).

When urine samples (10 ml) were spiked with [^{3}H]PGE_2 and processed exactly as the plasma samples, 91 ± 1% (mean ± S.D., n = 4) of the added radioactivity was recovered in the ethyl acetate fraction. However, when large volumes (400 ml) of urine were processed through a single column (6 ml, C_{18}), the recovery of PGE_2 was poor (16—31%); moreover, the columns tended to clog from the accumulation of amorphous sediment resulting in a significant prolongation of the time required to carry out an extraction. This problem of column obstruction could be lessened but not totally eliminated by centrifuging the urine prior to application to the column. Thus, our experience would indicate that this method is not very efficient for recovering trace amounts of prostaglandin from large urine volumes. On the other hand, when large volumes of protein-free buffer (400 ml) were processed through a single column (6 ml, C_{18}), the recovery of added PGE_2 was usually greater than 80%. These results would suggest that this method might be of utility in extracting trace amounts of prostaglandin from large volumes of buffer such as might be obtained from an organ perfusion.

This improved sample preparation procedure using Baker disposable columns is simpler and more rapid than our previous procedure which involved solvent extraction and chromatography on silicic acid [9, 10]. We have investigated this procedure most extensively in regard to TXB_2 and PGE_2. When whole blood was allowed to clot and the serum (which contained high levels of TXB_2) was serially diluted and processed through the Baker columns and analyzed by RIA, parallelism could be demonstrated. Processing a buffer blank through the Baker columns results in insignificant assayable levels of immunoreactive material (< 5 pg/ml of TXB_2 or PGE_2). This is in contrast with previous attempts employing Amberlite XAD-2 to avoid solvent extraction [1]. With Amberlite columns, significant amounts of polymer leach from the resin resulting in inhibition of antigen—antibody binding thereby precluding its use

in processing RIA samples even though this extraction method is efficient, reproducible and rapid. However, XAD-2 Amberlite can be used in preparing prostaglandin samples for GC—MS.

Powell [11] and Müller et al. [12], utilizing Sep-Pak C_{18} cartridges from Waters Assoc. (Milford, MA, U.S.A.), have described sample extraction procedures similar to that reported here. Müller et al. [12] combined the use of normal- and reversed-phase columns to extract PGE_2, $PGF_{2\alpha}$ and PGE-M from plasma and urine prior to GC—MS. Powell [11] used reversed-phase C_{18} columns to extract several arachidonic acid metabolites from plasma, urine and tissue homogenates. Neither of these previous studies reported recovery data for 5-HETE, 12-HETE or LTB_4. Such procedures employing the Waters Sep-Pak and the Baker Extraction System illustrate the potential and growing use of this approach as an alternative to solvent extraction in processing biological samples containing arachidonic acid metabolites.

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