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SEPARATION AND QUANTITATIVE DETERMINATION OF RADIOLABELED PROSTAGLANDINS, THROMBOXANES, 6-KETO-PROSTAGLANDIN $F_{1\alpha}$ AND OTHER ARACHIDONIC ACID METABOLITES PRODUCED IN BIOLOGICAL MATERIAL

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SUMMARY

Evaluation of several thin-layer chromatographic procedures for the separation of various labeled arachidonic acid metabolites (including 6-keto-prostaglandin $F_{1\alpha}$) produced in the biological system is described. Manual scanning and autoradiography of the plates developed by two-dimensional thin-layer chromatography was also done for locating the radioactivities due to arachidonic acid metabolites other than thromboxane B_2 and the classical prostaglandins ($PGF_{2\alpha}$, PGE_2, and PGD_2).

INTRODUCTION

Arachidonic acid (AA) is rapidly metabolized in the biological system by two main enzymatic pathways initiated separately by cyclooxygenase and lipoxygenase [1]. Cyclooxygenase converts arachidonic acid into the pivotal prostaglandin endoperoxides, which are then transformed into thromboxane A_2 (TxA_2), the classical prostaglandins, 12-hydroxy-heptadecatrienoic acid (HHT), malondialdehyde (MDA), and prostacyclin (PGI_2). Thromboxane A_2 is the main product in blood platelets while PGI_2 is chiefly produced by blood vessels. Thromboxane A_2 synthesis in platelets is usually accompanied by a simultaneous production of thromboxane B_2 (TxB_2), HHT and MDA. Prostacyclin is very unstable in the biological system and is readily converted non-enzymatically into 6-keto-$PGF_{1\alpha}$ whose determination gives a quantitative idea of the level of PGI_2. In platelets, lipoxygenase produces 12(*S*)-hydroperoxy-5,8,10,14-eicosatetraenoic acid (HPETE) which is subsequently converted into the corresponding hydroxy compound (HETE). In blood platelets, the lipoxygenase pathway has recently been shown to produce three more hydroxy

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fatty acids, viz. 8,11,12-trihydroxyeicosa-5,9,14-trienoic acid [2], 8,9,12-trihydroxyeicosa-5,10,14-trienoic acid (THETA) [2], and 10-hydroxy-11,12-epoxyeicosa-5,8,14-trienoic acid (EPHETA) [3].

In leukocytes a new family of conjugated lipoxygenation products are formed from AA, which serves as a substrate for 5-lipoxygenase. These are collectively called leukotrienes, viz. 5,12-diHETE or leukotriene B_4 and the leukotrienes C_4, D_4 and E_4 (LTC_4, LTD_4 and LTE_4) [4]. The latter three compounds make up what is known as SRS-A, a slow-reacting substance of anaphylaxis, an important mediator of hypersensitivity responses that causes slow contraction of smooth muscle during allergic reaction.

The relative formation of prostaglandins, thromboxanes, hydroxy fatty acid (HPETE) and prostacyclin in the cardiovascular system is of great interest as these compounds exhibit opposite biological effects, i.e. whereas TxA_2 stimulates [5], PGE_1 [6], PGD_2 [7], HPETE [8] and PGI_2 [9] inhibit platelet aggregation/release reaction. There are several reports found in the literature on the chromatographic separation of prostaglandins [10—17]. In these reports information is lacking on the separation of prostaglandins from thromboxanes probably because thromboxanes were discovered either at the same time or later after the reports were published. Later some reports were published in which separation of TxB_2 from other prostaglandins and their quantitation have been described [18—20]. In the latter reports, TxB_2 was separated from the prostaglandins by unidimensional thin-layer chromatography (TLC).

There is one report which deals with the differential separation of thromboxanes from prostaglandins by two-dimensional TLC and the radiolabeled products were determined semiquantitatively with the help of a radioscanner [21]. Later, with the discovery of prostacyclin, its separation as a stable metabolite (6-keto-$PGF_{1\alpha}$) from other AA metabolites which are formed concomitantly in the biological system, and determination were desirable. Thus, as several closely related biologically active compounds are formed from AA, it is necessary to evaluate the various methods of resolution of such compounds and finally develop a convenient schedule for the separation and quantitation of these compounds useful for routine work. In the present report, therefore, methods are described for the separation and quantitation of labeled AA metabolites produced in human blood platelets and in the aorta and lung of rat.

EXPERIMENTAL

Authentic PGE_2, $PGF_{2\alpha}$, PGD_2, TxB_2 and 6-keto-$PGF_{1\alpha}$ were obtained from ONO Pharmaceutical Company, Osaka, Japan; HHT and HETE were from Dr. D.H. Nugteren, Unilever Research, Vlaardingen, The Netherlands, as generous gifts. Unlabeled AA was purchased from Nu Chek Prep., Elysian, MN, U.S.A.; and [1-^{14}C]AA (spec. act. 58.4 mCi/mmol) was from The Radiochemical Centre, Amersham, Great Britain. All organic solvents and chemicals were of analytical grade and were purchased from Merck, Darmstadt, G.F.R. Silica gel G plates (0.25 mm, 20×20 cm) were prepared in our laboratory and were activated (1 h) before use. In some separations, prepared plates (DC-Alufolien, Kieselgel 60 F_{254}, 20×20 cm, Merck) were used. Authentic PGs, TxB_2, HHT, and HETE were used as methanolic solutions. 6-Keto-$PGF_{1\alpha}$ was dissolved in

methanol—ethyl acetate (3:1, v/v). AA was dissolved in toluene. The solutions were stored at −20°C.

Blood platelets

Preparation of platelet suspension, incubation and extraction

Platelet suspension was prepared as described earlier [22]. Platelet suspension (300 μl, 10^8 platelets) was incubated with labeled arachidonate (final concentration 20 μM in total incubation volume of 315 μl) at 37°C for 10 min. Extraction of the incubation mixture was done in ethyl acetate and the solvent evaporated. The residue was dissolved in 200 μl of ethanol and used for TLC separation.

Chromatographic separation of AA metabolites

AA metabolites were separated by TLC. Reference standards (3—5 μg) were first applied on the plate followed by incubation extract containing the radiolabeled AA metabolites as one single spot (diameter ca. 7 mm) with the help of a Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland). Plates were developed using the ascending technique in tightly closed rectangular tanks (Desaga, Heidelberg, G.F.R.) at room temperature (21°C). Chromatography tanks were not equilibrated with the solvent mixture as this has been reported to result in less efficient separation [23]. During TLC developments, a solvent run of ca. 17 cm from the application point to the solvent front was maintained.

Separation of ^{14}C-labeled HHT, HETE, and AA. This was conveniently done by unidimensional TLC resolution of 25 μl of the ethanolic extract by solvent system I (chloroform—methanol, 90:3, v/v). Spots were visualized in iodine, marked, scraped off and their radioactivity counted.

Unidimensional TLC separation of ^{14}C-labeled AA metabolites. This was achieved by resolving 25 μl of the ethanolic extract in the three solvent systems II (chloroform—methanol—acetic acid, 90:8:6, v/v), III (benzene—dioxane—acetic acid, 40:20:2, v/v), and IV (diethyl ether—methanol—acetic acid, 90:1:2, v/v). Classical PGs and TxB_2 were used as standard reference compounds. Their spots were visualized in iodine, marked, scraped off, and their radioactivity counted. The remaining portion of the developed part of the plate was divided into several areas corresponding to 1.2 cm along the direction of the development × 2.5 cm (breadth), scraped off and counted.

Separation of ^{14}C-labeled $PGF_{2\alpha}$, PGE_2, PGD_2 and TxB_2 by two-dimensional TLC. On the left-hand bottom corner of a plate were spotted authentic samples of the above reference standards. On the same spot were placed 25 μl of ethanolic solution of incubation extract. The plate was developed in two sets of solvent systems, II and III or II and IV. Spots were visualized in iodine, marked, scraped off and their radioactivity counted.

Separation of ^{14}C-labeled HHT, HETE, AA, $PGF_{2\alpha}$, PGE_2, PGD_2 and TxB_2 on a single TLC plate by a three-solvent development. TLC plates spotted as described above were subjected to three successive developments in solvents I, II, III or I, II, IV. The details of this method have been reported earlier [24].

Autoradiography of TLC plates after two-dimensional development. On the

left-hand bottom corner of the TLC plates (DC Alufolien, Kieselgel 60 F_{254}, 20 × 20 cm) were placed reference standards (PGs and TxB_2) followed by 25 μl of the ethanolic extract. The plates were developed in solvents II and III or II and IV as described for ^{14}C-labeled AA metabolites, the spots were visualized in iodine, and marked. The plates were then exposed to X-ray film for 7 days. Areas containing strong radioactivity produced marks on the X-ray plates.

Manual scanning of TLC plate after two-dimensional development. Two TLC plates were used. For two-dimensional development, the reference compounds and the extracted material were spotted as described above. One of the plates was developed in solvents II and III, and the other plate in solvents II and IV. The spots were visualized in iodine, marked, scraped off and counted. The remaining portion of the plate was divided into 1.3 cm × 1.3 cm areas which were carefully scraped off and their radioactivity counted.

Aorta and lung (rat)

Preparation of homogenate, incubation and extraction

Incubation medium in the case of lung consisted of 500 μl of lung homogenate, that for aorta consisted of a piece of aorta in 250 μl of phosphate buffer (0.06 *M*, pH 7.4, 2 m*M* EDTA). Lung homogenate was prepared in phosphate buffer (1:4, w/v). The two preparations were incubated with labeled AA (final concentration for aorta 25 μM, for lung 12.5 μM) for 15 min at 37°C. The incubation mixture was extracted with ethyl acetate after addition of saline and acidification (with HCl) to pH 3.0 as described for platelets. The residue obtained was dissolved in 250 μl of methanol—ethyl acetate (4:1, v/v) in the case of aorta, and in 300 μl of methanol—ethyl acetate (2:1, v/v) in the case of lungs.

TLC separation of ^{14}C-labeled $PGF_{2\alpha}$, PGE_2, PGD_2, TxB_2 and 6-keto-$PGF_{1\alpha}$

This was achieved by two-dimensional TLC using solvents II and III as described for platelets. A relatively greater amount of 6-keto-$PGF_{1\alpha}$, as reference standard compared to other PGs was needed for being visualized on the plate in iodine.

RESULTS

Blood platelets

Unidimensional TLC. Table I gives the results for the separation of ^{14}C-labeled PGs and TxB_2 in 25 μl of the incubation extract. The three solvent systems II, III and IV were used. In solvent systems II and IV the PGs and TxB_2 were resolved, whereas solvent system III did not separate TxB_2 from PGE_2. Manual scanning of the entire length of the developed part of the plate showed that radioactivity was distributed along the entire length of the plate and even in areas between $PGF_{2\alpha}/TxB_2$, TxB_2/PGE_2 and PGE_2/PGD_2. High radioactivity areas, as expected, were located a little below the solvent front. Just above the application point, considerable radioactivity was found. In each solvent system, repeated separations of a certain incubation extract gave close values for the resolved PGs and TxB_2; the values, however, were different in different solvent systems.

TABLE I

TLC SEPARATION OF LABELED AA METABOLITES FORMED IN HUMAN PLATELETS FROM LABELED AA BY UNIDIMENSIONAL DEVELOPMENT IN THREE SOLVENT SYSTEMS*

Values are reported as cpm (mean ± S.D.) of three separations. Exactly equal amounts of the same incubation extract were resolved in different solvent systems (unidimensional).

	Sample 1		Sample 2		
Metabolite	II	III	II	III	IV
$PGF_{2\alpha}$	589 ± 81	467 ± 16	467 ± 50	370 ± 69	337 ± 39
TxB_2	19018 ± 388 }	19436 ± 1311	4522 ± 117 }	4664 ± 99	3511 ± 164
PGE_2	1308 ± 107 }		539 ± 20 }		384 ± 28
PGD_2	768 ± 68	1160 ± 175	351 ± 12	438 ± 17	630 ± 43

*II, chloroform—methanol—acetic acid (90:8:6, v/v); III, benzene—dioxane—acetic acid (40:20:2, v/v); IV, ether—methanol—acetic acid (90:1:2, v/v).

TABLE II

UNIDIMENSIONAL TLC SEPARATION OF HHT AND HETE FORMED IN BLOOD PLATELETS FROM LABELED AA

Solvent system I: chloroform—methanol (90:3, v/v).

Metabolite	cpm (mean ± S.D.)*
HHT	3198 ± 136
HETE	9776 ± 926
Application spot**	10983 ± 282

*Three separations.

**Due to classical PGs + TxB_2 + phospholipids + other known and unknown AA metabolites.

TABLE III

TLC SEPARATION OF LABELED AA METABOLITES FORMED IN HUMAN PLATELETS BY TWO-DIMENSIONAL DEVELOPMENT

Values are reported as cpm. For each sample, an exactly equal amount of the incubation extract was resolved in the different solvent systems.

	Sample I		Sample 2	
Metabolite	II and III*	II and IV**	II and III*	II and IV**
$PGF_{2\alpha}$	258 ± 22	204	130 ± 16	103
TxB_2	18055 ± 1045	17365	4266 ± 159	3972
PGE_2	443 ± 88	478	230 ± 28	198
PGD_2	484 ± 45	562	166 ± 24	167

*Three separations (mean ± S.D.).

**Two separations.

Separation of AA, HHT and HETE from PGs and TxB_2. Though the major part of the radioactivity was localized in AA, HHT and HETE and also in areas between HHT/HETE, and HETE/AA, a substantial amount of radioactivity (>1000 cpm per 1.2 cm × 2.5 cm area) was found distributed along the entire length, right from the application point to the HHT spot (Table II).

Two-dimensional TLC. Table III gives the separation results for the PGs and TxB_2 resolved by two sets of solvents. As can be seen, the values for TxB_2 are quite close to those obtained in unidimensional TLC. In the case of prostaglandins $F_{2\alpha}$, E_2 and D_2, the values were reduced by about 35—50% of those obtained in unidimensional TLC. The values for PGs in the two solvent sets were acceptably close.

Manual scanning after two-dimensional TLC. Besides TxB_2, the major radioactivity was found distributed in the top right-hand corner of the plate in areas 7.5 cm × 10.5 cm (solvents II and III), and 8.0 cm × 9.5 cm (solvents II and IV) (Fig. 1). The radioactivity contained in these areas should mainly be due to excess AA, HHT, HETE and other known and unknown metabolites of AA.

Autoradiography of TLC plates after two-dimensional development. Major radioactivity was localized in the spot due to TxB_2, and at the right-hand top corner area, confirming the results obtained by manual scanning (Fig. 2). Plates

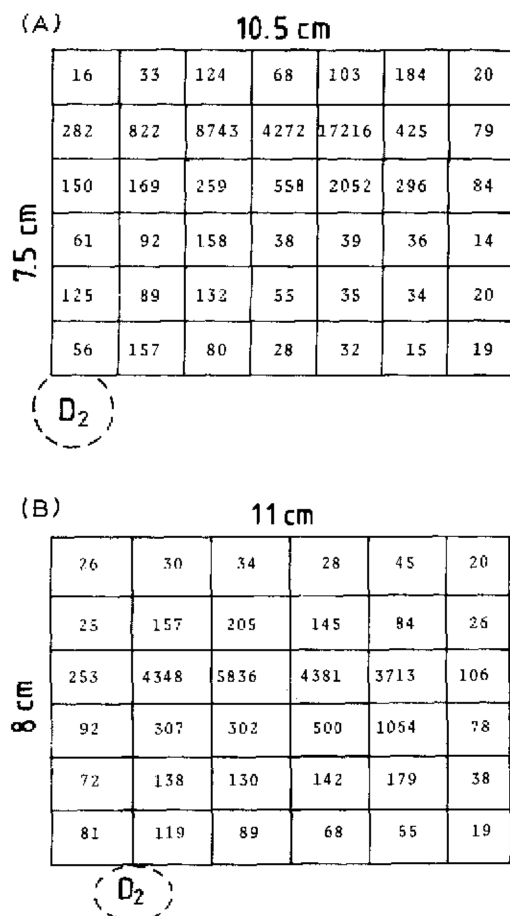

Fig. 1. Manual scanning of the top right-hand corner area of the TLC plate after two-dimensional development. (A) Developed successively in solvents II and III; area scanned 10.5 cm × 7.5 cm. (B) Developed successively in solvents II and IV; area scanned 11 cm × 8 cm. The values are cpm in the respective area. Location of PGD_2 is also shown.

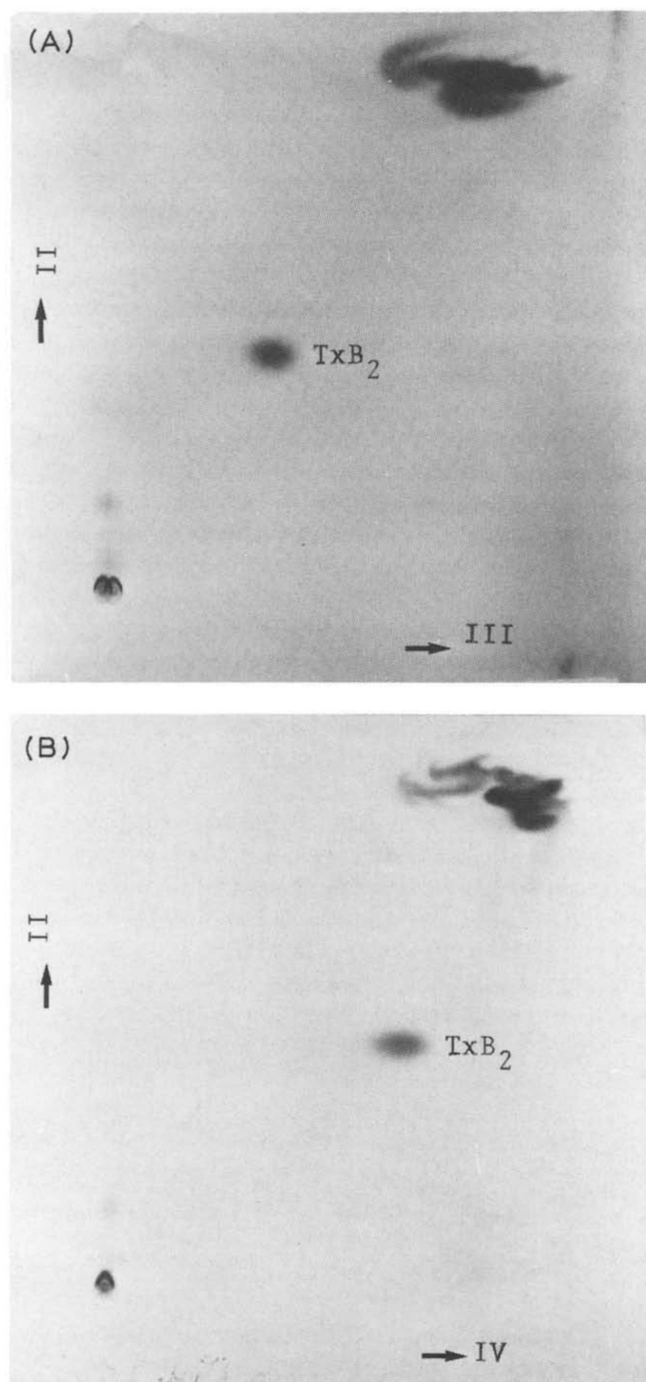

Fig. 2. Autoradiography of the TLC plate developed successively in solvents II and III (A), and solvents II and IV (B). The spot in the middle is due to TxB_2. Areas of high radioactivity are seen in the top right-hand corner of the plate.

developed unidimensionally in solvents II and III, were also subjected to autoradiography. The radioactivity distribution confirmed that ascertained by manual scanning.

Aorta and lung (rat)

Chromatographic separation of ^{14}C-labeled $PGF_{2\alpha}$, PGE_2, PGD_2, TxB_2 and 6-keto-$PGF_{1\alpha}$. Separation of these AA metabolites was achieved by two-dimensional TLC (Fig. 3) and their amounts were calculated after taking into consideration the recovery factors. Results are shown in Table IV.

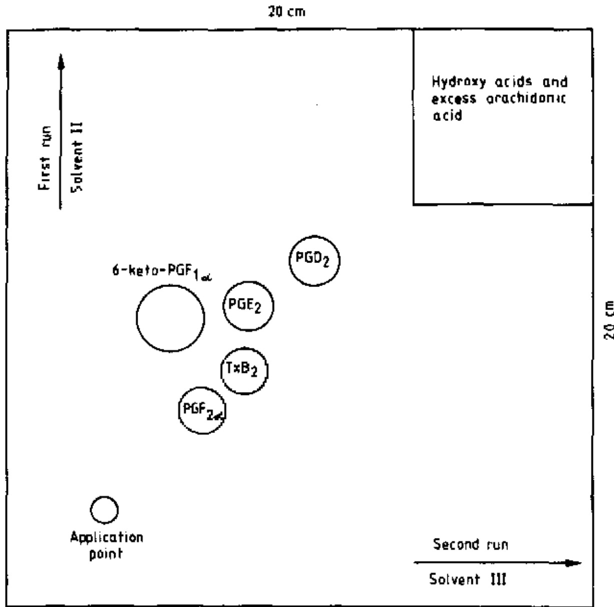

Fig. 3. Schematic presentation of the separation of prostaglandins, thromboxane, 6-keto-prostaglandin $F_{1\alpha}$ and hydroxy acids by two-dimensional TLC.

TABLE IV

SEPARATION OF LABELED AA METABOLITES FORMED BY AORTA RINGS AND LUNG HOMOGENATE OF RAT BY TWO-DIMENSIONAL TLC

Values (mean ± S.D.) are reported as picomoles per aorta piece (13.2 mg) and as picomoles per 500 μl of lung homogenate of three separations which were achieved by developing the plate in solvents II and III in succession.

Tissue	AA metabolite				
	6-keto-$PGF_{1\alpha}$	$PGF_{2\alpha}$	TxB_2	PGE_2	PGD_2
Aorta	76 ± 5	5 ± 2	13 ± 3	5 ± 1	4 ± 2
Lung	214 ± 16	18 ± 3	148 ± 10	21 ± 2	18 ± 3

DISCUSSION

Several solvent systems have been described in the literature for the complete resolution of the PGs and TxB_2 by unidimensional development. Repeated separation of AA metabolites in an incubation extract by unidimensional development using a certain solvent system gives reproducible results. But separation results, with the exception of TxB_2, are not the same with other solvent systems in the case of classical PGs for which varying values are obtained. This necessitates a further resolution. Two-dimensional TLC was found suitable because separation by different sets of solvent systems yields comparable values for TxB_2 and classical PGs. While no appreciable change was observed in the TxB_2 counts obtained in the two-dimensional TLC when compared with those obtained in the unidimensional development, the same for the classical PGs were reduced by 35—50% of the values obtained with unidimensional TLC. Manual scanning of the plate after two-dimensional development showed that the radioactivity was mainly localized in the spot due to TxB_2, in the application spot and also in the spots of HHT, HETE and AA. Other areas of radioactivity were observed too. The chemical characterization of the unknown radioactivities evidently requires further study.

Although a three-solvent development of the same plate is suitable for the separation of AA, HHT, HETE, TxB_2 and PGs [24], one may best utilize the separation schedule described in this paper by a combination of unidimensional development (for the separation of AA, HHT, and HETE) and two-dimensional development (for the separation of PGs and TxB_2) if one can afford a little more material for the separation. Further, this combined procedure will require one extra TLC plate per 5 or 6 samples (for the separation of AA, HHT and HETE). Moreover, for the complete resolution of a mixture containing 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) with the other AA metabolites mentioned above, a combination of unidimensional and two-dimensional separation is suitable. For the separation of 6-keto-$PGF_{1\alpha}$ from other AA metabolites, it is necessary to acidify the incubation medium with hydrochloric acid (instead of the most frequently used citric acid) prior to extraction with ethyl acetate because acidification with citric acid leads to the development of multiple spots of 6-keto-$PGF_{1\alpha}$ on the TLC plate [25].

In prostaglandin research one is often interested in the endogenous synthesis of the biologically active AA metabolites which are usually determined by either bioassay or radioimmunoassay. These methods, though very useful for quantitative assay, have limitations in the costs involved if one would like to estimate several of the AA metabolites. Formation of AA metabolites from the labeled precursor may give an insight into the endogenous biosynthesis of all the known AA metabolites and more so of their relative proportions. This can be achieved by labeling the AA pool of the membrane phospholipids and subsequently subjecting them to hydrolysis by a phospholipase reaction [26]. This is possible only when a suitable separation schedule is available. For this purpose our method is convenient and reproducible giving reliable prostaglandin values which do not change on further TLC. Manual scanning of the TLC plate has shown that TxB_2 is the main cyclooxygenase product in the blood platelets.

and that areas of unknown radioactivity are localized close to those of the hydroxy acids (HHT, HETE).

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