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ENZYMATIC SYNTHESIS OF TRITIUM LABELLED EICOSANOIDS

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SUMMARY

Methods for the synthesis of tritium labelled prostaglandins, thromboxanes, and hydroxy-derivatives of eicosapolyenoic acids using mono-, two-, and three-enzyme systems have been developed. Optimization of the eicosanoid syntheses with respect to the concentrations of enzymes and eicosapolyenoic acid, time, and temperature of incubation are reported. It is demonstrated that a number of compounds can be produced by enzymatic modifications of prostaglandins and thromboxane. Procedures for the preparation of twenty tritium labelled compounds are described.

Key Words: Tritium Labelled Eicosanoids; Enzymatic Synthesis; Arachidonic Acid; Prostaglandins; Thromboxane.

INTRODUCTION

Eicosanoids are constituent parts of many human regulatory systems. They are formed by enzymatic oxygenation of C_{20} polyunsaturated fatty acids, mainly, by prostaglandin (PG) synthase and lipoxygenases. Studies of the metabolism and mechanisms of action of eicosanoids require extensive use of isotopically labeled compounds. The latter can be obtained either by direct incorporation of the label in the nonlabeled compounds (for review see [1]) or by enzymatic conversion of previously labeled eicosapolyenoic acids to the desired compounds. This paper is devoted to the development of the latter approach. Virtually any isotopically labeled eicosanoid with a natural configuration can be obtained this way.

The enzymatic synthesis of a number of tritium labelled PGs has been previously accomplished in our laboratory and elsewhere [2-11]. In general, crude enzymatic preparations from sheep vesicular glands containing PGE forming enzymes were used. In this paper data

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on the optimization of the syntheses of isotopically labeled eicosanoids using different partially purified enzymes are presented.

RESULTS AND DISCUSSION

1. Use of prostaglandin synthase for the synthesis of isotopically labeled eicosanoids

A number of compounds can be obtained from isotopically labeled eicosapolyenoic acids with the use of only the enzyme PG synthase. This enzyme catalyzes oxygenation of eicosapolyenoic acid to PGH, which is extremely unstable in an aqueous medium [12]. Prostaglandins D, E, and F_α, 12-hydroxyheptadecapolyenoic acid (HHPA), and malonic dialdehyde are the products of its decomposition.

1.1. Synthesis of prostaglandins H and F_α

Due to PGH instability in aqueous media its preparation relies on a short reaction time and rapid transfer of the reaction products to a non-aqueous medium. The kinetics of PGH accumulation during the course of the reaction is described by a curve with a maximum, the time corresponding to the maximum PGH yield being denoted τ_{opt}. The PGH maximum yield and τ_{opt} values depend on both eicosapolyenoic acid and PG synthase concentrations (Fig. 1). It was established that the PGH maximum yield is determined by the ratio of the above parameters and the dependence was found to be similar to that obtained for the degree of substrate conversion [14]. The PGH yield was shown to be virtually independent on the incubation temperature within 15-50 °C while τ_{opt} decreased with increasing incubation temperature. Table 1 (part A) shows the established optimum conditions for PGH and PGF_α synthesis from arachidonic (AA) and thymnodonic (TA) acids.

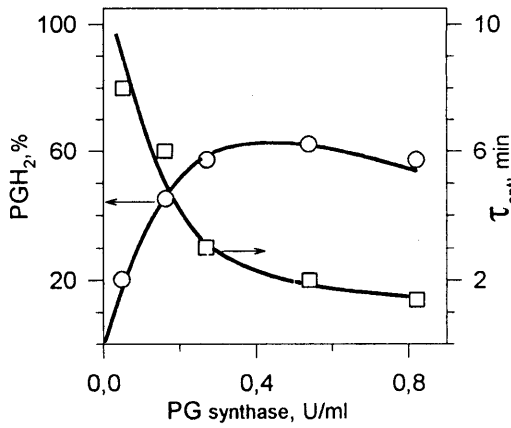


Fig.1 Enzymatic synthesis of PGH_2 from arachidonic acid. The effect of PG synthase concentration on the yield of the product (-o-) and τ_{opt} (-□-). AA – 100 μM; 25 °C.

The amount of PGH_2 was estimated by the formation of its stable reaction product $PGF_{2\alpha}$ upon addition of $SnCl_2$ to the reaction mixture [13].

Table 1. Optimum conditions for the enzymatic synthesis of isotopically labeled eicosanoids

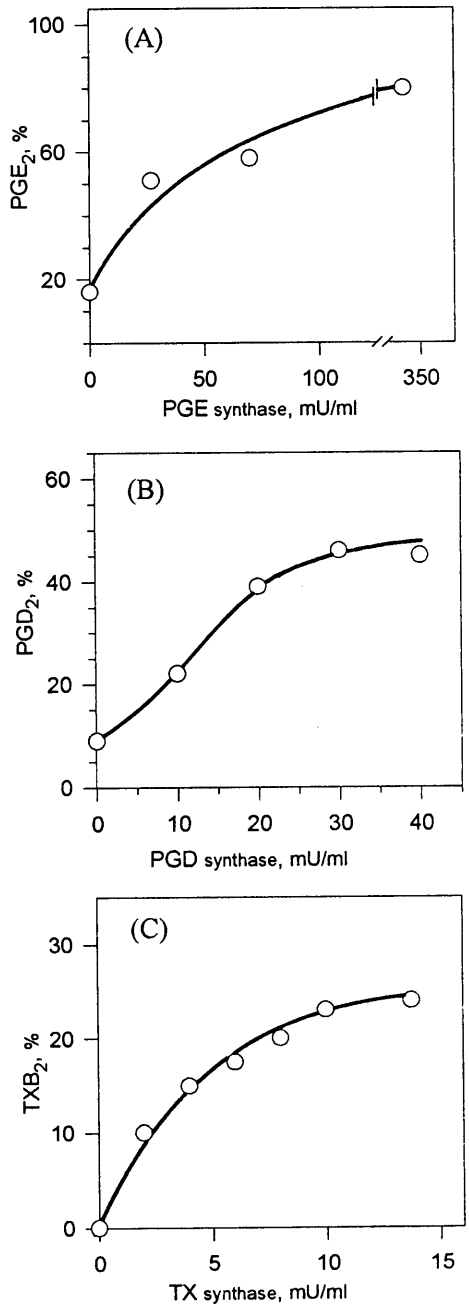
Synthesis conditions	Initial compounds	
	Arachidonic acid	Thymnodonic acid
A. Synthesis of prostaglandins H and F_α		
1. Ratio of the initial concentrations of eicosapolyenoic acid and PG synthase, nmol/U	250	80
2. Incubation temperature, °C	15-50	15-50
3. Incubation time, min	10.0 (15 °C)	7.0 (15 °C)
	1.3 (30 °C)	2.5 (30 °C)
	0.5 (50 °C)	1.0 (50 °C)
B. Two-enzyme synthesis of prostaglandins and thromboxane		
1. Ratio of the initial concentrations of eicosapolyenoic acid and PG synthase, nmol/U	750	60
2. Initial concentration of PGH-converting enzyme, mU/ml	150*	150*
3. Incubation temperature, °C	20-25	20-25
4. Incubation time, min	40	40
* The values shown were calculated for eicosapolyenoic acid concentration of 100 μM		

1.2. Synthesis of eicosanoids by nonenzymatic destruction of prostaglandin H

The ability of PGH to undergo spontaneous transformation to other products can be exploited for the syntheses of isotopically labeled compounds without the use of any special enzymes. When eicosapolyenoic acids and PG synthase were co-incubated in different buffers the ratios of PGH stable products PGD, PGE, and PGF_α were also different. For example, in 0.1 M tris-buffer pH 9.5 it was 0.12:0.23:0.64, while in 50 mM potassium-phosphate buffer pH 7.4 it was 0.19:0.39:0.42. Besides, acidification of the reaction mixture to pH 3 and keeping for 1 h after 1-2 min incubation led to an increase in the HHPA fraction up to 0.70.

2. Two-enzyme synthesis of isotopically labeled eicosanoids

Application of the mono-enzyme system does not allow production of compounds which can not be obtained by spontaneous PGH decomposition. Besides, the reaction conditions ensuring formation of preferentially one product can not be established. These problems were overcome with the help of two-enzyme systems. Enzymes specifically converting PGH to certain compounds (PGH isomerases) were used in parallel with PG synthase:



PGE synthase was used for the synthesis of PGE, PGD synthase for the synthesis of PGD, and thromboxane (TX) synthase for the synthesis of TXB and HHPA.

The main results for the optimization of the two enzyme synthesis of eicosanoids are the following.

1. Concentrations of PG synthase and eicosapolyenoic acid should be calculated bearing in mind the optimum ratio of these components: 750 and 60 nmol of eicosapolyenoic acid per unit of PG synthase activity with AA and TA as substrates, respectively.

2. To achieve a high yield of the desired product high concentrations of PGH-converting enzymes are required. They can vary from 15 to 150 mU/ml, depending on the initial concentration of eicosapolyenoic acid (see Fig. 2). This requirement is due to the necessity of making the rate of PGH enzymatic conversion considerably higher than the rate of its spontaneous decomposition [15].

3. The enzymatic reaction time is no greater than the time of PGH decomposition (30-40 min).

Fig.2 Two-enzyme synthesis of prostaglandins E_2 (A), D_2 (B), and thromboxane B_2 (C) from arachidonic acid. The effect of PGH-converting enzyme concentration on the yield of the product. **(A).** AA - 100 μM; PG synthase - 0.38 U/ml; 60 min; 25 °C. **(B).** AA - 50 μM; PG synthase - 0.2 U/ml; 60 min; 25 °C. **(C).** AA - 25 μM; PG synthase - 0.12 U/ml; 60 min; 25 °C.

4. The temperature dependence of the yield of PGE and other eicosanoids in two-enzyme synthesis is characterized by an optimum between 20 and 25 °C.

5. The yield of the desired product was increased when better purified preparations of the enzymes were used. This fact can be accounted for by sorption of eicosapolyenoic acid on ballast proteins in poorly purified preparations thus making it inaccessible for PG synthase. The sorption is reversible and eicosapolyenoic acid can be extracted from the proteins by organic solvents. Another reason is the ability of nonspecific proteins to convert PGH to different by-products [16].

6. The yield of the desired product can be increased 1.5-2-fold by separate addition of PG synthase and PGH-converting enzyme to the reaction mixture. The optimum incubation time of eicosapolyenoic acid with PG synthase prior to the introduction of PGH-converting enzyme is 1.5-2 min. This time corresponds to the time of the maximum PGH accumulation during the PG synthase reaction, i.e. τ_{opt} (see Fig. 1).

Table 1 (part B) summarizes the results for the optimization of the two-enzyme synthesis of PGs and TX.

Table 2. Prostaglandin modification by rabbit liver prostaglandin reductase and human platelets 15-hydroxy-prostaglandin dehydrogenase

A. Reduction of tritium labelled prostaglandins by prostaglandin reductase		**B.** Oxidation of tritium labelled prostaglandins by prostaglandin dehydrogenase	
Initial compound	Degree of conversion, %	Initial compound	Degree of conversion, %
PGE_1	95	PGD_2	36
PGD_2	93	PGB_1	30
PGE_2	90	PGB_2	22
15-keto-13,14-dihydro-PGE_2	82	PGE_1	15
PGE_3	82	$PGF_{1\alpha}$	10
PGA_1	13	PGE_2	9
PGA_2	13	$PGF_{2\alpha}$	9
PGB_1	7		
13,14-dehydro-Δ^{12}-PGJ_2	6		
PGB_2	4		
15-keto-13,14-dihydro-PGD_2	<2		

3. Synthesis of eicosanoids by enzymatic modification of prostaglandins and thromboxane

A number of prostanoids can be obtained by modification of certain functional groups in a precursor molecule. The advantages of using enzymes are obvious: they allow one-stage modification of small amounts of the initial compound. Modified eicosanoids were synthesized via the following reactions: i)- by reduction of the keto group of the cyclopentane ring (Table 2, Part A); ii)- by oxidation of the C15(S)-hydroxy group and reduction of the Δ^{13} double bond of PGs (Table 2, Part B), and iii)- by dehydration of the thromboxane C11-hydroxy group.

4. Synthesis of 12-hydroxy- and 12-hydroperoxy-derivatives of eicosapolyenoic acids

Incubation of 12-lypoxygenase from human platelets with AA led to formation of 12(S)-hydroxy-5(E),8(Z),10(Z),14(Z)-eicosatetraenoic acid (12-HETE) and a number of unidentified products. 12(S)-hydroperoxy-5(E),8(Z),10(Z),14(Z)-eicosatetraenoic acid (12-HpETE) was not detected among the reaction products. This is accounted for by the presence of high peroxidase activity in the enzymatic preparation. It was found that indometacin addition to the reaction mixture resulted in suppression of peroxidase and accumulation of 12-HpETE. With an indometacin concentration of 200 μM 12-HpETE was the main reaction product. It was also shown that performing the 12-lipoxygenase reaction in the presence of some reducing agents led to a considerable increase in the 12-HETE yield and a decrease in the amount of by-products formed (see Table 3). The most marked effect was observed when dithiothreitol or reduced glutathione were used.

Table 3. Effect of reducing agents on the 12-HETE synthesis

Reducing agent (200 μM)	Degree of AA conversion	Yield of 12-HETE, %
Dithiothreitol	0.59	51
Glutathione	0.60	49
L-Cysteine	0.73	44
L-Triptophane	0.84	36
Aniline	0.85	34
Control	**0.85**	**34**
L-Adrenaline	0.83	32
L-Methionine	0.83	32
β-NADH	0.66	31
Phenol	0.74	29
NADPH	0.73	21

EXPERIMENTAL

Reagents and materials

[5,6,8,9,11,12,14,15(n)-^{3}H] Arachidonic, [8,9,11,12,14,15(n)-^{3}H] digomo-γ-linolenic (DHLA), and [5,6,8,9,11,12,14,15,17,18(n)-^{3}H] thymnodonic acids were prepared as described earlier [7, 17]. Tritium labeled prostaglandins A_1, A_2, B_1, B_2, D_2, 15-keto-13,14-dihydro-PGD_2, and 13,14-dihydro-Δ^{13}-PGJ_2 were obtained according to the procedures previously reported in [5,7,8-11]. Arachidonic and digomo-γ-linolenic acids were obtained from Fluka Chemie AG products. Thymnodonic acid was kindly supplied by Dr. N.A. Latyshev (Institute of Marine Biology, Vladivostok, Russia). Prostaglandins were obtained from the Institute of Chemistry (Tallinn, Estonia). Hemin, Lubrol PX, glucoso-6-phosphate, indometacin, and L-amino acids were obtained from Sigma Chemical Co.; reduced glutathione, β-NADH, and NADPH from Reanal (Hungary); L-adrenalin from Serva Feinbiochemica GmbH; glucoso-6-phosphate dehydrogenase from Fluka Chemie AG; β-NAD and NADP from E. Merck Darmstadt. Other reagents and solvents were chemically pure products. PGH_2 and PGH_3 was obtained as described in [18].

Preparation of enzymes

PG synthase and PGE synthase preparations (microsomes and DEAE fraction) were prepared from sheep vesicular glands as described by F.J. Van der Ouderaa [19] and T. Miyamoto [20], respectively. PGD synthase preparation was produced from rat brain as described in [11]. TX synthase preparation (DEAE fraction) was obtained from human platelets according to [21]. For the production of the enzyme preparation with 15-hydroxy-PG-dehydrogenase and 15-keto-PG-Δ^{13}-reductase activities from human platelet (Blue Sepharose fraction) the procedure described by T. Watanabe [22] was utilized. Rabbit liver cytosole fraction in 50 mM potassium phosphate buffer pH 7.4 was used as a preparation of 11-hydroxy-TX-dehydrogenase. The preparation of keto-PG-reductase (ammonium sulfate fraction) was isolated from rabbit liver according to [23].

Gas chromatography analysis demonstrated that enzymatic preparations used in the current studies had no admixtures of free fatty acids.

Identification and analysis of eicosanoids were performed as previously described [14]. Separation of the products was conducted on Kieselgel F254 (Fluka Chemie AG) and Silufol (Kavalier, Czechia) silicagel plates. Analysis of radiochromatograms was performed using a Berthold 2027 radioactivity scanner (Germany).

The following TLC standard solvent systems were used.

A. ethylacetate/hexane/isopropanol/acetic acid - 15:9:1:0.5, v/v/v/v.
B. benzol/ethyl acetate/acetic acid - 80:20:0.2, v/v/v.
C. chloroform/methanol/acetic acid - 85:14:1, v/v/v.
D. chloroform/methanol/acetic acid - 90:9:1, v/v/v.
E. benzol/ethyl acetate/acetic acid - 50:50:2, v/v/v.

Preparation of tritium labelled eicosanoids

[5,6,8,9,11,12,14,15-^{3}H] Prostaglandin H_2 (1) and [5,6,8,9,11,12,14,15,17,18-^{3}H] Prostaglandin H_3 (2)

1.14 ml of 20 mM potassium phosphate buffer (pH 7.8) containing phenol (3 μmol) and hemine (2.4 nmol) were added to 23 mCi [^{3}H] AA (150 Ci/mmol) in 50 μl of ethanol. After 5 min of pre-incubation at 25 °C 60 μl of PG synthase preparation (0.52 U) was introduced to the reaction mixture and after continuous stirring at 25 °C for 1.5 min 40 μl of 2 M citric acid solution and 15 ml of ethyl acetate precooled to -20 °C were added to the mixture. The organic phase was then separated and dried with anhydrous sodium sulfate. The yield of (1) was about 70 % (TLC in the solvent system A at -20 °C; R_f 0.54).

(2) was obtained similarly starting from [^{3}H] TA (200 Ci/mmol). 6.9 U of PG synthase were used for 20 mCi of [^{3}H] TA. The yield of (2) was about 45 % (TLC in the solvent system A at -20 °C; R_f 0.52).

Purification of (1) and (2) was carried out by TLC in the solvent system A at -20 °C. The obtained compounds were stored in dry acetone under argon at -20 °C.

12-Hydroxy-[5,6,8,9,11,12-^{3}H]-heptadecatrienoic acid (3) was produced from [^{3}H] AA (190 Ci/mmol). Synthesis conditions were the same as in the production of (1) except that the reaction mixture was incubated 1 hour more at 25 °C after the reaction was stopped by cytric acid addition (with pH shift down to 3). The yield of (3) was 35 % (TLC in the solvent system B; R_f 0.21). Purified (3) displayed a typical spectrum with the maximum at 232 nm.

[5,6,8,9,11,12,14,15-^{3}H] Prostaglandin $F_{2\alpha}$ (4) and
[5,6,8,9,11,12,14,15,17,18-^{3}H] Prostaglandin $F_{3\alpha}$ (5)

1.16 ml of 20 mM potassium phosphate buffer (pH 7.8) containing Lubrol PX (0.5 %, w/v), L-adrenalin (2.6 μmol), and hemine (2.6 nmol) were added to 38 mCi [^{3}H] AA (150 Ci/mmol) in 30 μl of ethanol. After 5 min pre-incubation at 25 °C 0.14 ml of PG synthase preparation (1.2 U) were added to the reaction mixture and after 1.5 min incubation with vigorous agitation 0.22 ml of $SnCl_2$ solution in methanol (60 mg/ml) were added. The yield of (4) was 63 % (TLC analysis in the solvent system C; R_f 0.41).

(5) was produced in a similar way. 5 U of PG synthase were used for 62 mCi of [^{3}H] TA (180 Ci/mmol). $SnCl_2$ was added 3.5 min after the start of the enzymatic reaction. The yield of (5) was 27 % (TLC analysis in the solvent system C; R_f 0.38).

[5,6,8,9,12,14,15,17,18-^{3}H] Prostaglandin D_3 (6) was produced from [^{3}H] TA (200 Ci/mmol) according to the method described in [11], except that the ratio of the amounts of PG synthase and eicosapolyenoic acid was 10 times larger. The yield of (6) was 25 % with respect to radioactivity (TLC analysis in the solvent system D; R_f 0.36).

[5,6,8,11,12,14,15-^{3}H] Prostaglandin E_2 (7),
[8,11,12,14,15-^{3}H] Prostaglandin E_1 (8), and
[5,6,8,11,12,14,15,17,18-^{3}H] Prostaglandin E_3 (9)

1.025 ml of 20 mM potassium phosphate buffer (pH 7.4) containing L-adrenalin (2.6 μmol), reduced glutathione (2.6 μmol), and hemine (2.6 nmol) were added to 35 mCi of [^{3}H] AA (150 Ci/mmol) in 40 μl of ethanol. After 5 min pre-incubation at 23 °C PG synthase (340 mU) and PGE synthase (93 mU) preparations in the volume of 135 μl were introduced to the reaction mixture and incubated for 1 h at 23 °C. The yield of (7) was 77 % (TLC analysis in the solvent system D; R_f 0.30).

(8) was produced from [^{3}H] DHLA (100 Ci/mmol) with the use of the procedure described above. The yield of (8) was 62 % (TLC analysis in the solvent system D; R_f 0.28).

In the case of (9) the method underwent modifications in concentrations of the initial compound and enzymes. 4.3 U of PG synthase and 0.1 U of PGE synthase were used for 25 mCi of [^{3}H] TA (250 Ci/mmol). The yield of (9) was 36 % (TLC analysis in the solvent system D; R_f 0.30).

[8,11,12,14,15-^{3}H] Prostaglandin $F_{1\alpha}$ (10),
[5,6,8,11,12,14,15-^{3}H] Prostaglandin $F_{2\alpha}$ (11), and
[5,6,8,11,12,14,15,17,18-^{3}H] Prostaglandin $F_{3\alpha}$ (12)

Syntheses of (10) and (11) were conducted the same way as (7) and synthesis of (12) as that of (9) except that after 1h incubation of eicosapolyenoic acids with the PG and PGE synthases at 32 °C NADP was added to the reaction mixture to a final concentration of 0.5 mM, glucoso-6-phosphate to 5 mM, and a preparation of rabbit liver PG-ketoreductases to 0.27 U/ml and the incubation continued for another hour at 32 °C. The yields of (10), (11), and (12) were about 60, 75, and 40 %, respectively, according to the TLC analysis in the solvent system C (R_f appr.0.4).

15-keto-13,14-dihydro-[8,11,12,14-^{3}H]-Prostaglandin E_1 (13) and
15-keto-[8,11,12,14-^{3}H]-Prostaglandin B_1 (14)

1 ml of reaction mixture containing NADP (0.2 μmol) and a preparation of human platelet 15-hydroxy-PG-dehydrogenase (10 U) in 0.1 M tris buffer pH 9 were added to 3 mCi of (8). Upon 1.5 h incubation at 32 °C with continuous stirring glucoso-6-phosphate (5 μmol) were added and incubation continued for a further 30 min. The yield of (13) was 17 % (TLC analysis in the solvent system D; R_f 0.60).

(14) was obtained from [^{3}H] PGB_1 (45 Ci/mmol) the same way but without addition of glucoso-6-phosphate to the reaction mixture. The yield of (14) was about 60 % (TLC analysis in the solvent system E; R_f 0.56).

[5,6,8,9,11,12,14,15-^{3}H] Thromboxane B_2 (15),
12-hydroxy-[5,6,8,9,11,12-^{3}H]-Heptadecatrienoic acid (3),
12-hydroxy-[8,9,11,12-^{3}H]-Heptadecadienoic acid (16), and
[5,6,8,9,11,12,14,15,17,18-^{3}H] Thromboxane B_3 (17)

4.57 ml of 20 mM potassium phosphate buffer pH 7.4 containing L-adrenalin (10 μmol) and hemine (10 nmol) were added to 76 mCi of [^{3}H] AA (150 Ci/mmol) in 100 μl of ethanol. After 5 min pre-incubation at 25 °C 0.3 ml of PG synthase preparation (2.8 U) and after 1 more minute 3.5 ml of TX synthase preparation (0.14 U) were added to the reaction mixture and the incubation continued for 30 min. The yield of (15) and (3) was about 35 and 25 % according to TLC analysis in the solvent system D for (15) (R_f 0.21) and in the system B for (3), respectively.

(16) was produced from [^{3}H] DHLA (100 Ci/mmol) using the same procedure. The yield of (16) was about 70 % (TLC analysis in the solvent system B; R_f 0.22).

For the preparation of (17) modifications in concentrations of the initial compound and enzymes were introduced to the above procedure. 8.6 U of PG synthase and 0.2 U of TX synthase were used for 60 mCi of [^{3}H] TA (180 Ci/mmol). The yield of (17) was about 20 %; (TLC analysis in the solvent system D; R_f 0.19).

11-dehydro-[5,6,8,9,12,14,15-^{3}H]-Thromboxane B_2 (18)

Reaction mixture containing 0.5 ml of 0.2 M potassium phosphate buffer pH 7.5, 0.1 ml of 250 mM solution of β-NAD, and 0.4 ml of enzyme preparation of rabbit liver 11-hydroxy-TX-dehydrogenase were added to 1 mCi of (15) (100 Ci/mmol). Incubation was conducted for 1 h at 32 °C. The yield of (18) was about 80 % (TLC analysis in the solvent system D; R_f 0.31).

12-hydroxy-[5,6,8,9,11,12,14,15-^{3}H]-Eicosatetraenoic acid (19) and 12-hydroperoxy-[5,6,8,9,11,12,14,15-^{3}H]-Eicosatetraenoic acid (20)

1.5 ml of 25 mM tris buffer pH 7.7 containing reduced glutathione (2 μmol) were added to 15 mCi of [^{3}H] AA (190 Ci/mmol). After 5 min preincubation at 32 °C 0.5 ml of 12-lipoxygenase preparation (20 mU) was added to the reaction mixture and incubated for 2 hours at 32 °C with continuous stirring. The yield of (19) was 83 % (TLC analysis in the solvent system B; R_f 0.31). Purified (19) displayed a typical spectrum with the maximum at 236 nm.

For the production of (20) the described procedure underwent the following modifications: the buffer used in the reaction mixture contained no reduced glutathione and the reaction was conducted in the presence of indometacin (200 μM). The yield of (20) was about 70 % (TLC analysis in the solvent system B; R_f 0.44).

Isolation and purification of tritium labelled eicosanoids

After incubation the pH of the reaction mixture was brought down to 3 with 2 M citric acid and radioactive products were extracted either by ethyl acetate (three times with 4-fold volumes) or with Sep Pac C_{18} Cartridge (Millipore Associates) as described in [25]. The organic phase was then separated and dried with anhydrous sodium sulfate. Purification was conducted by TLC (see above) or HPLC as described in [24].

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