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DETERMINATION OF PROSTAGLANDIN PRECURSORS IN FROG TISSUE USING SELECTED-ION MONITORING IN GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS

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SUMMARY

Eicosatrienoic acid, eicosatetraenoic acid (arachidonic acid), and eicosapentaenoic acid are unsaturated fatty acids that are possible precursors for prostaglandin synthesis in amphibians. These compounds have been quantitatively identified in tissues of the American bullfrog by using methods of selected-ion monitoring for fatty acid methyl esters. Results from comparison of yield for four methylation techniques showed that boron trifluoride in methanol gave the best yield and reproducibility. Resolution values of 1.2 and 2.7 for these methyl esters were achieved by using a cyanosilicone liquid phase, and the limits of detection based on ion 79.1 a.m.u. were 0.5 ng absolute. These acids were extracted and methylated with overall recoveries of 47, 37 and 28%, respectively. Methyl esters were detected in extracts of all tissues in concentration ranges 12 to 912 ng/μl of extract, or 30 to 2810 μg/g of tissue for 0.1- to 1.3-g samples.

INTRODUCTION

Prostaglandins (PGs) are a family of biologically active compounds that are synthesized from polyunsaturated fatty acid precursors released from plasma membrane phospholipids[1]. In well-described mammalian pathways, arachidonic acid (AA) ($C_{20:4}$, ω-6) is utilized in the synthesis of bisenoic prostaglandins (*e.g.*, PGE_2). However, production of monoenoic (*e.g.*, PGE_1) or trienoic (*e.g.*, PGE_3) prostaglandins from eicosatrienoic acid ($C_{20:3}$,ω-6) or eicosapentaenoic acid ($C_{20:5}$,ω-3), respectively, might also contribute, in some organisms, to biochemical or physiological regulation[2,3]. Eicosapentaenoic acid (EPA) and eicosatrienoic acid (ETA) are readily incorporated into tissue phospholipids of mammals if diets are supplemented with these fatty acids[4,5]. For example, Eskimos have elevated levels of EPA from their fish-based diet, and AA is predominant in mammals with meat-based diets[6]. EPA is largely unused in mammals for synthesis of trienoic prostaglandins and acts as a

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competitive inhibitor in prostaglandin synthesis involving AA[7,8]. Prostaglandin synthesis has not been extensively studied in non-mammalian vertebrates. Although utilization of AA and EPA particularly has been suggested from recent evidence[9], the natural availability of these precursors in tissues of amphibians has been unknown.

Gas chromatographic (GC) methods have been used in separation of ETA, AA and EPA on both packed[10] and capillary columns[11], and most analyses have been based on comparison of relative retention times and detection by ionization detectors[12–14]. Although liquid phases used in these analyses were ester-based materials, such as diethylene glycol succinate, cyanosilicone phases with properties of high temperature stability and low bleed have also been used[15,16]. In this paper, quantitative techniques based on GC–mass spectroscopy (MS) with selected-ion monitoring (SIM) have been developed and used for determining ETA, AA and EPA in representative tissues of the American bullfrog, *Rana catesbeiana*. In addition, heart and urinary bladder tissue from the related leopard frog, *R. pipiens* were analyzed for comparative purposes. Several derivatization procedures have been examined quantitatively for use with these unsaturated fatty acids.

EXPERIMENTAL

Instrumentation

A Hewlett-Packard model 5880A gas chromatograph was equipped with a splitless inlet, a flame ionization detector (FID), and a 0.2 mm I.D. × 25 m OV-101 wall-coated open tubular (WCOT) column. Conditions of analysis were: initial temperature, 150°C; final temperature, 250°C; temperature-programme rate, 4°C/min; final time, 10 min; detector and injection-port temperature, 250°C; attenuation, $16 \cdot 10^{-12}$ A full scale; carrier gas, helium; average linear velocity 20 cm/sec; and splitless time, 0.5 min.

A Hewlett-Packard model 5992A GC–MS instrument was equipped with splitless inlet, glass-jet separator, and 0.2 mm I.D. × 30 m OV-101 WCOT column for use in scanning GC–MS; the chromatographic conditions were as described above. The MS conditions were: lower mass range, 45 a.m.u.; upper mass range, 500 a.m.u.; scan rate, 330 a.m.u./sec; electron multiplier voltage, 2000 V; and solvent time out, 3 min. Other MS conditions were optimized by using Autotune software provided by the manufacturer.

A 2 mm I.D. × 2.5 m glass column containing 1.8663 g of 10% SP-2330 on Supelcoport (100–120 mesh; Supelco, Bellefonte, PA, U.S.A.) was installed in the GC–MS system for SIM analysis. Chromatographic conditions were: oven temperature, 250°C (isothermal); injection-port temperature, 250°C; and helium carrier gas flow-rate, 24 ml/min. Conditions for MS during SIM analysis were: ions monitored, 55.0, 67.0, 79.1, 91.1, 320.1 and 318.1 a.m.u.; dwell time, 166 msec; electron-multiplier voltage, 2000 V; and solvent time out, 8 min. Response in SIM analysis was characterized through preparation of calibration curves for the methyl esters of ETA, AA and EPA at approximate mass levels of 50, 25, 12, 5 and 0.5 ng.

Derivatization study

The yield (%) and reproducibility of derivatization of four methylation techniques were determined for ETA, AA and EPA. These methods involved use of

diazomethane, BF_3 in methanol, Methylute (Alltech Assoc., Deerfield, IL, U.S.A.) or Methyl-8 (Alltech). Stock solutions of each fatty acid [8,11,14-eicosatrienoic acid (Sigma, St. Louis, MO, U.S.A.), 5,8,11,14-eicosatetraenoic acid (AA) (Sigma) and 5,8,11,14,17-eicosapentaenoic acid (Analabs, North Haven, CT, U.S.A.)] were prepared at 50 mg/ml in ethanol and stored at −20°C. Five replicate samples were prepared from stock solutions for use in each derivatization method so that final concentrations were *ca.* 1000 ng/μl; the absolute amount of acid varied between 25 and 500 μg, depending on the procedure.

(a) Diazomethane was prepared by using 133 mg of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG; Aldrich, Milwaukee, WI, U.S.A.) freshly prepared in 0.5 ml of water and placed in an apparatus (Aldrich) containing 3 ml of anhydrous ethyl ether (Mallinkrodt, St. Louis, MO, U.S.A.). Diazomethane was generated for 45 min at 1°C after 0.6 ml of 5 *N* NaOH had been slowly added to the MNNG solution. A 2-μl volume of stock solution was placed in a 5.0-ml Wheaton mini-vial (Southland Cryogenics, Carrolton, TX, U.S.A.), and evaporated to dryness in a stream of nitrogen. Then 0.9 ml of the ether solution was added, and the vial was capped and gently shaken. After 60 min, the solution was evaporated to dryness and reconstituted in 100 μl of hexane (Burdick and Jackson, Muskegon, MI, U.S.A.) for analysis by GC.

(b) A 10-μl volume of sample was placed in a mini-vial and evaporated to dryness. Then 2 ml of BF_3 in methanol (Supelco) were added and the vial was sealed and stored at 60°C for 20 min in a Temp-Blok Module Heater (Scientific Products, Tempe, AZ, U.S.A.). Deionized water (2.0 ml) was added, and the mixture was vigorously shaken for 1 min with 1 ml of hexane. The aqueous phase was removed with a pipet, and hexane was condensed to 500 μl and analyzed by GC.

(c) A 12.5-μl volume of a new sample solution (2 mg/ml) was evaporated to dryness in a mini-vial. Methylute (25 μl) was added, and the mixture was injected directly into the GC system.

(d) New sample solution (150 μl) was evaporated to dryness in mini-vials. Methyl-8 (300 μl) was added to each sample, and each mixture was heated at 60°C for 10 to 15 min in sealed mini-vials before analysis by GC.

Since methyl arachidonate (Sigma) was the only methyl ester of known purity available, and since weight-response factors for FID are affected only slightly or not at all by the numbers (*e.g.*, 2 *vs.* 3, or 3 *vs.* 4) of double bonds in a particular chain length fatty acid[17], yield was quantified for each acid *vs.* methyl arachidonate.

In a second derivatization study, solutions of AA and ETA at 100 ng/μl and 10 ng/μl were derivatized by using BF_3 in methanol (the preferred method) to measure any effects of concentration on the percentage yield.

Extraction of tissues

Rana catesbeiana or *R. pipiens* (Nasco Biological, Fort Atkinson, WI, U.S.A.) were pithed, and tissues were quickly excised and weighed. They were extracted with 40% KOH solution in methanol with 0.1 ml of hydroquinone solution (1 mg/ml) as described by Kent *et al.*[18]. The mixture was heated under reflux at 90°C for 1 h, acidified to pH 3.0 and extracted three times with 6 ml of diethyl ether. The combined extracts were evaporated to dryness under nitrogen, and the residues were methylated with 2.0 ml of BF_3 in methanol as described above. Final volumes for these samples were 1.0 ml in hexane.

"Spiking" studies

Absolute recovery efficiencies were determined for tissue extraction and the entire analytical method. Samples of tissue were divided into equal parts, and one part was "spiked" with 100 μg of each acid from stock solutions. Samples were separately extracted, derivatized and analyzed by SIM as described above. Comparison of the actual increase in concentration with the expected increase corrected for derivatization efficiency gave the yield from extraction. Values were determined for three replicates for ETA, AA and EPA.

RESULTS AND DISCUSSION

Results from studies on yield from the four methylation techniques with EPA, AA and ETA are listed in Table I. Values for the percentage yield with use of BF_3 in methanol were generally as good or better than those for the other methods, including the use of diazomethane. On the basis of percentage yield and acceptable standard deviations, BF_3 in methanol was adopted for use in subsequent analyses. The percentage yield for AA at two other concentration levels with this reagent were: 10 ng/μl, 67; and 100 ng/μl, 69.8. Thus, under our experimental conditions, the efficiency of derivatization of AA did not vary substantially within the concentration range expected in these tissue extracts. The value for derivatization of ETA at 100 ng/μl also remained nearly constant at 57.5%; however, efficiency was lost at the 10-ng level, for which the value was 31%.

TABLE I
YIELD FOR METHYLATION TECHNIQUES

Reagent	*Yield (%)*		
	ETA	*AA*	*EPA*
Diazomethane	57.7 ± 8.5	40.9 ± 6.0	56.7 ± 6.6
BF_3–methanol	56.0 ± 1.3	77.4 ± 7.9	59.1 ± 1.7
Methyl-8	50.0 ± 1.8	43.8 ± 3.2	57.8 ± 3.1
Methylute	24.4 ± 6.7	5.7 ± 0.5	7.0 ± 0.9

The chromatographic performance of the GC–MS system is shown in Fig. 1a as a SIM plot from the analysis of a standard solution of the methyl (Me) esters of ETA, AA and EPA. Retention times and specific retention volumes at 200°C for these compounds were: Me-ETA, 10.7 min, 1.86 l/g; Me-AA, 11.8 min, 1.52 l/g; and Me-EPA, 15.1 min, 1.94 l/g. Reproducibility of retention time was better than ±0.1 min, and values for resolution were 1.2 and 2.7 for Me-ETA/Me-AA and Me-AA/Me-EPA, respectively. MS patterns of these acids were similar, and major ions common to each were 55.0 a.m.u., C_4H_7; 67.0 a.m.u., C_5H_7; 79.1 a.m.u., C_6H_7; and 71.1 a.m.u., C_7H_7. These four most abundant ions were chosen for monitoring in SIM analysis, together with the molecular ions for Me-ETA (320.1 a.m.u.) and Me-AA (318.1 a.m.u.). Because four ions were monitored and ratios of abundance values were used, instances of false identification based only on single-ion monitoring were reduced in tissue analysis. Results from the SIM analysis of two tissue extracts are

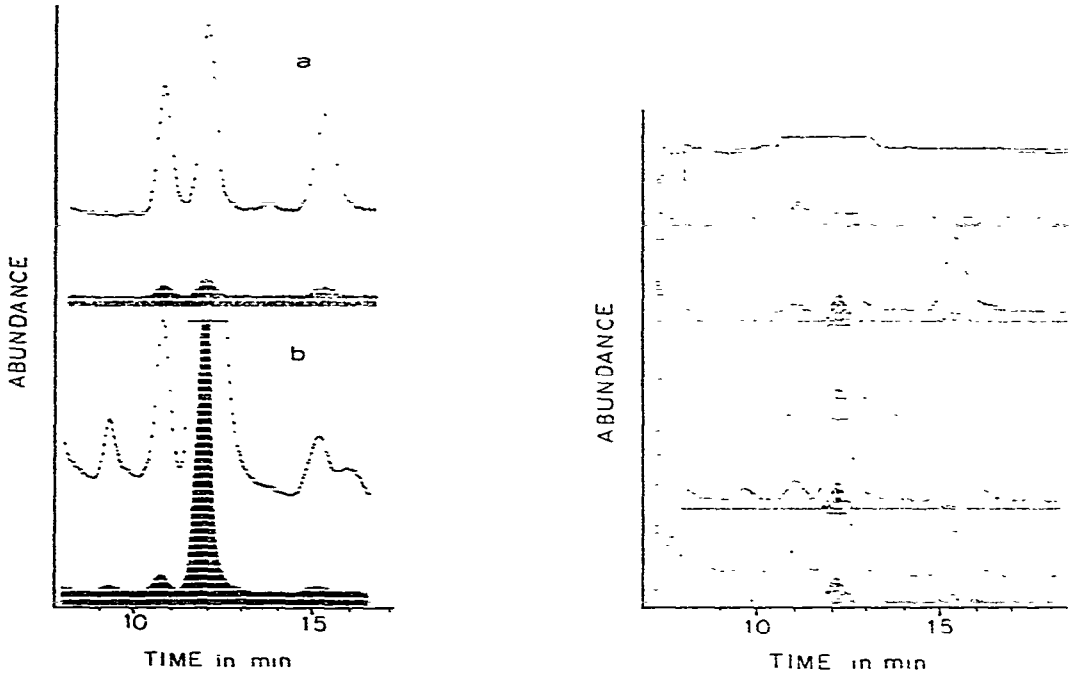

Fig. 1. Plots from SIM analysis of (a) standard and (b) tissue extract. Abundance values are the sum of the values for six ions. Order of elution is Me-ETA, Me-AA and Me-EPA on 10% SP-2330.

Fig. 2. Plots of individual ions from SIM analysis of lung-tissue extract.

shown in Figs. 1b and 2. In Fig. 1b, the summed abundance of the total ions monitored is plotted from analyses of tissue extract and standard solution for comparison of retention behavior. Retention times for the standards and for several large components in the extract were the same. Resolution of SIM data into individual ions is shown in Fig. 2 as plots of individual ion abundance *vs.* time from analysis of another similar tissue extract. Full scale (FS) values, which indicate relative concentrations, clearly show the ion 79.1 a.m.u. as being the ion of choice for the best limits of detection. The major ions for each methyl ester are also seen in these plots. The probability of false identification is further reduced by positive matches between mass spectra from this sample and standard spectra. Also present in these plots is a molecular ion for Me-ETA. Throughout these analyses, interferences when present were detected in plots of ion 55.0 a.m.u. and, in diminishing degree, in plots of ions of higher mass. Consequently, the ion 79.1 a.m.u. was chosen for quantitative analysis. Calibration curves for SIM analysis using 79.1 a.m.u. are shown in Fig. 3. The response of the entire GC–MS system is linear on a log–log plot, and the sensitivities for Me-ETA, Me-AA and Me-EPA are comparable.

However, absolute limits of detection will vary very slightly and were between 0.3 to 0.7 ng for all methyl esters. Instrumental blanks consisting of 2 μl of fresh hexane were used periodically to determine residual contaminations from syringe, septum and column. No components were ever detected in these blanks. Procedure blanks, which were treated as samples (but without tissue) were used to determine

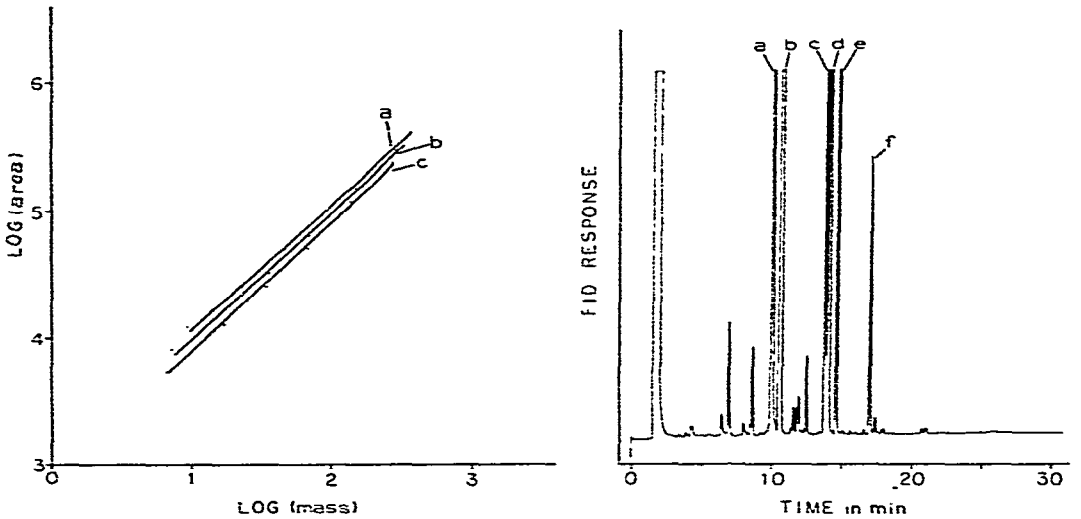

Fig. 3. Calibration curve for Me-ETA (c), Me-AA (b) and Me-EPA (a) based on the 79.1 a.m.u. ion from SIM analysis. Values for mass are in nanograms absolute.

Fig. 4. Chromatogram from GC–FID analysis of lung-tissue extract. For identities of major components (a–f) see Table II.

levels of contamination from glassware, vials, extraction apparatus and sample handling. Small amounts (less than 1 ng/μl) of Me-AA were detected in these blanks; other methyl esters were detected, but below 1 ng for 1-μl injections. Although the origins of these small amounts of contaminants are unknown, by comparison with the natural abundance (12 to 912 ng/μl), these errors in quantitative analyses are negligible.

Extracts of these tissues were relatively free from interfering components, and results from GC–FID analysis of the extract of the lung sample are shown in Fig. 4. Less than twenty major components were detected and were tentatively identified by using mass spectra. These compounds, which are listed in Table II, were present in every extract and thus may also be expected to be widely abundant. The three unsaturated fatty acids were not completely resolved on this capillary column.

TABLE II

COMPOUNDS IDENTIFIED USING MASS SPECTRA FROM ANALYSIS OF LUNG TISSUE

Reference to Fig. 4	*Retention time (min)*	*Compound*	*Molecular ion (a.m.u.)*	*Major ions (a.m.u.)*
a	12.8	Me-Palmitoleate	268	55, 74, 69, 236
b	13.5	Me-*n*-Hexadecanoate	260	74, 87, 143
c	16.4	Me-Linoleate	294	55, 67, 71, 95
d	16.6	Me-Oleate	296	55, 69, 74, 264
e	17.2	Me-*n*-Octadecanoate	298	74, 87, 143, 267
f	19.3	Me-Arachidonate	N.D.	79, 67, 91, 55

Results from the "spiking" study showed average extraction efficiencies and standard deviations for each acid were: ETA, 84 ± 14; AA, 48 ± 6; and EPA, 48 ± 1%. When corrected for average derivatization efficiency the overall net recoveries and standard deviations* for the entire analytical procedure were: ETA, 47 ± 8; AA, 37 ± 6; and EPA, 28 ± 10%. Results from analysis of several types of tissue are given in Table III. These values are expressed in units of μg/g and have been corrected for overall recovery efficiency. Every tissue extract contained more than 2 ng/μl of ETA, AA and EPA. However, with values lower than 10 ng/μl, automated integration failed, and triangulation was unreliable for peaks of this low abundance, especially with large full-scale values for Me-AA. All three unsaturated fatty acids were found in representative frog tissues. The data suggests a natural abundance, in order of

TABLE III

ANALYSIS OF PROSTAGLANDIN PRECURSORS IN FROG TISSUES

Sample No.	*Tissue type*	*Weight (g)*	*Concentration in extract (ng/μl)*			*Concentration in tissue (μg/g)*		
			ETA	*AA*	*EPA*	*ETA*	*AA*	*EPA*
1	Heart	0.96	<5	525	<5	Tr*	1330	Tr
2	Heart**	1.08	12	759	219	30	2280	870
3	Heart**	0.42	<5	347	95	Tr	2680	970
4	Heart**	0.74	20	457	110	60	1670	530
5	Bladder	0.29	<5	174	<5	Tr	2110	Tr
6	Bladder	0.13	<5	87	<5	Tr	2350	Tr
7	Bladder	0.56	20	416	<5	60	2810	Tr
8	Bladder**	0.66	13	263	100	50	1290	650
9	Bladder**	0.18	<5	93	110	Tr	1670	2620
10	Bladder**	0.18	<5	79	75	Tr	1420	1780
11	Gastric mucosa	1.26	71	912	117	130	2150	360
12	Gastric mucosa	1.26	63	870	107	120	2050	330
13	Gastric mucosa	1.34	53	794	72	100	1920	230
14	Lung	0.99	46	603	59	130	2140	280
15	Atria	0.19	<5	96	<5	Tr	1640	Tr
16	Atria	0.14	<5	96	<5	Tr	2220	Tr
17	Ventricle	0.38	<5	200	<5	Tr	1560	Tr
18	Ventricle	0.51	<5	437	30	Tr	2540	230
19	Conus arteriosus	0.15	<5	87	<5	Tr	1880	Tr
20	Conus arteriosus	0.10	<5	17	<5	Tr	550	Tr

* Tr indicates trace amount, insufficient for quantification.

** Pooled tissue from five to seven frogs (*Rana pipiens*); all other values are individual tissues from *R. catesbeiana*.

* $s_{total} = \bar{x}(\sqrt{s_1^2 + s_2^2})$ where $\bar{x}$ is average net recovery, s_1 is relative standard deviation of derivatization and s_2 is relative standard deviation of extraction. s_{total} is given in absolute standard deviation.

decreasing concentration: AA, EPA, ETA. Thus, AA is the most abundant prostaglandin precursor in *R. catesbeiana* tissues, and undoubtedly offers a major pool of substrate for bisenoic prostaglandin synthesis. The other fatty acid precursors are also available in significantly smaller quantities. These amounts are several orders of magnitude greater than the physiological concentrations of prostaglandins and could therefore be utilized for synthesis of monoenoic and trienoic prostaglandins. Interestingly, in the *R. pipiens* tissue examined, both AA and EPA represented major fatty acid components.

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