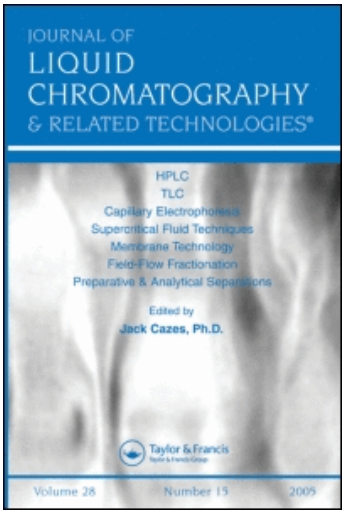

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 05 November 1999

To cite this Article Nomikos, T. N. , Antonopoulou, S. , Argyropoulos, D. and Demopoulos, C. A.(1999) 'DETERMINATION OF PLATELET-ACTIVATING FACTOR BY HPLC WITH FLUORESCENCE DETECTION', Journal of Liquid Chromatography & Related Technologies, 22: 9, 1331 — 1341
To link to this Article: DOI: 10.1081/JLC-100101735
URL: http://dx.doi.org/10.1081/JLC-100101735

J. LIQ. CHROM. & REL. TECHNOL., 22(9), 1331–1341 (1999)

DETERMINATION OF PLATELET-ACTIVATING FACTOR BY HPLC WITH FLUORESCENCE DETECTION

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ABSTRACT

A new method for the chemical determination of Platelet-Activating Factor (PAF) has been developed. The method is based on the derivatization of the PAF molecule into a fluorescent derivative, through a three step procedure and the determination of the obtained derivative with HPLC combined with a fluorescent detector. Initially, PAF is converted to hepta-fluorobutyroyl- derivative which is hydrolyzed with water. The hydrolyzed derivative of PAF reacts with 7-methoxycoumarin-4-acetic acid to yield a fluorescent derivative of PAF, which is subjected to HPLC analysis on a reverse C_{18} column and measured with a fluorescent detector. The range of linearity and the detection limit of the method has been detected. The method is linear up to 10 ng PAF with a detection limit of 0.3 ng, at a signal to noise ratio above 3. This new method was successfully applied to human blood after its extraction and purification by a published method. Finally, the entire method has also been applied to standard phospholipids.

INTRODUCTION

Platelet-activating factor (PAF), chemically characterized as 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine,[1] is considered to be one of the most potent endogenous chemical mediators of allergic and inflammatory reactions.[2] A wide variety of cell types produce PAF in very low concentrations, either in basal levels or upon stimulation.[3,4,5] PAF has also been detected in various body fluids.[6, 7, 8, 9]

Because of its potency and diverse biological actions on cells and tissues, PAF has been implicated in the pathogenesis of many diseases such as asthma, anaphylaxis, allergy, ischemia.[10] The accurate and rapid, routine determination of PAF in body fluids or tissues is absolutely necessary in order to clarify the possible role of PAF in these diseased states and in normal physiological processes. A variety of biological, physicochemical, and immunological assays have been developed so far for the determination of PAF in biological fluids and other natural sources.

Biological assays such as platelet aggregation and degranulation,[11] although very sensitive, need cumbersome procedures to isolate human or rabbit platelets and to perform the biological assay.

Physicochemical methods, such as mass spectrometry and GC-MS,[12,13] have been extensively used for structure-proof analysis of PAF but they are not adequate for routine analysis because they require expensive equipment and tedious procedures for the derivatization to volatile compounds.

Finally, radioimmunoassays[14] are time-consuming, complex and have interference problems by other phospholipids due to the limited specificity of the antibodies against PAF.

This work attempts to make a new approach to PAF assay by a chemical method which includes a three step derivatization procedure of PAF to a fluorescent analog and its determination by HPLC and fluorescent detector. The present method does not require experimental animals and expensive equipment and is not complicated.

EXPERIMENTAL

Materials and Reagents

Disposable TechElut SPE C_{18} columns 500mg/6mL were purchased from HPLC technology (England). Silica gel G type 60 used for analytical TLC

plates was purchased from Merck (Darmstadt, Germany). All reagents were of analytical grade and purchased from Merck. HPLC solvents were purchased from F.E.R.O.S.A. (Barcelona, Spain). Lipid standards of HPLC grade were obtained from Supelco (Bellefonte, PA). Synthetic C_{16}-PAF was obtained from BACHEM (Bubendorf, Switzerland). [^{3}H]PAF (NET 910) was purchased from NEN (Dupont, Boston, MA). Heptafluorobutyric anhydrite, 7-methoxy-coumarin-4-acetic acid, 1,2-dimethoxyethane, N,N'-dicyclohexylcarbodiimide, were bought from Sigma (St. Louis, MO).

Instrumentation

Radioactivity was measured in a 1209 RackBeta-Flexivial β-Counter (LKB-Pharmacia, Turku, Finland). HPLC was done on a Hewlett-Packard HP Series 1050 liquid chromatography (Avondale, PA) equipped with a Rheodyne (Berkley, CA) 7125 loop valve injector (500 μL). A HP Series 1050 UV-VIS spectrophotometer as well as a Jasco (Tokyo, Japan) intelligent fluorescence detector were used. UV detection was performed at 208 nm and fluorescence was monitored at 330 nm excitation and 415 nm emission wavelengths. Both detectors were connected to a HP 3395 integrator-plotter. An absorption column, silica 25 cm x 4.6 mm (I.D.), H5, from Hichrom (Reading, Berkshire, U.K.) and a reverse phase column Nucleosil-300, C18 column 7μ, 250 x 4 mm (I.D.) from Analysentechnik (Mainz, Germany) were used. The flow rate was 1mL/min.

The Electrospray Mass Spectrometry (ESMS) was recorded on a Fisons VG Quattro instrument with a VG Biotech Electrospray source, having hexapole lens. Nitrogen 99.99% pure was used as the nebulizing and bath gas at flows of 20 and 150 $dm^3\ min^{-1}$, respectively. The samples were injected in the flow of solvent (10 μL/min) of a Varian 9012 solvent delivery system, via a Fisons interface with a Rheodyne 7125 injector. The capillary voltage was optimum at about 3.30 kV for positive ions. The high voltage lens potential was kept at 0.56 kV. The focus and skimmer lenses voltages were 40 and 45 V, respectively, in the majority of the measurements, as these values produced the highest peak intensities and minimum fragmentation. HPLC grade methanol/water (70:30, v/v) 0.01 M in ammonium acetate was used as solvent.

Chemical Determinations

Phosphorus was determined according to the method of Bartlett[15] and ester determination was performed according to the method of Renkonen.[16]

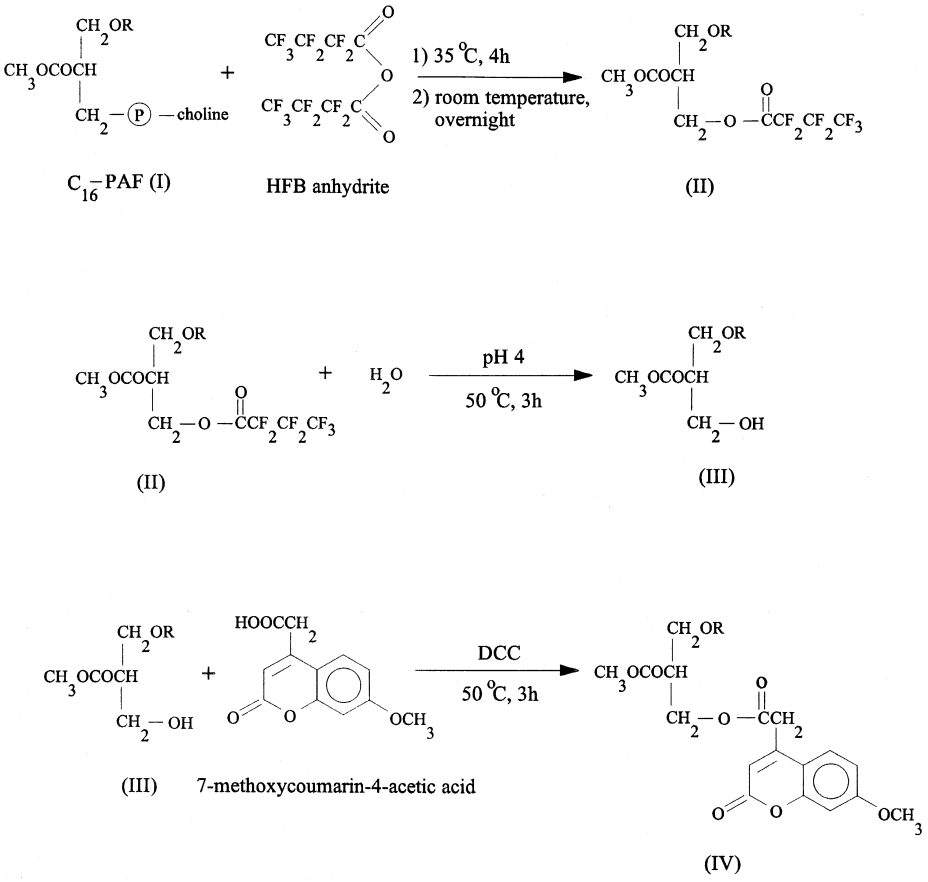

Figure 1. Preparation steps of 7-methoxycoumarin derivative of C_{16}-PAF (I); Heptafluorobutyroyl derivative of C_{16}-PAF (II); 1-O-hexadecyl-2-acetyl -*sn*-glycerol (III); 7-methoxycoumarin derivative of the 1-O-hexadecyl-2-acetyl-*sn*-glycerol (IV); Heptafluorobutyric (HFB); N,N'-dicyclohexyl carbodiimide (DCC).

RESULTS

Synthesis of Heptafluorobutyroyl Derivative (II) of C_{16}-PAF (I)

The synthesis of heptafluorobutyroyl derivative (II in Fig. 1) of C_{16}-PAF was performed according to the method of Satsangi et al.[17] Briefly, an amount of 2.71 μmol C_{16}-PAF (I in Fig. 1) in $CHCl_3$: CH_3OH (1:1, v/v) is taken to dryness in a siliconized 10 mL screw cap tube and 200 μL heptafluorobutyric

(HFB) anhydride is added. The tube is sealed with a Teflon-lined screw cap while the atmosphere above the reaction mixture is purged with nitrogen. The reaction mixture is heated at 35°C for 4 h with continuous stirring and the reaction is completed after incubation at room temperature overnight. Then, the reaction mixture is dried under a stream of nitrogen at room temperature and 1mL hexane and 1mL water are added. The compound II is partitioned in hexane layer. The aqueous layer is washed twice with 0.5 mL hexane which is added to the above hexane layer. The combined hexane layers are washed twice with 0.5 mL water, dried under a stream of nitrogen, and stored in a small volume of hexane.

The above procedure was also performed on approximately equal amounts of phosphatidylcholine (PC), lysophosphatidylcholine (LPC), lyso-PAF, and sphingomyelin (SM). Phosphorus determination in the aqueous layer obtained from PC derivatization, showed that the yield of the desired product was 90%.

The identification of compound II was based on its Rf (0.15) in TLC system, hexane:diethylether (70:30, v/v), proposed by Satsangi et al.[17] In addition, a TLC system was developed, namely hexane:diethylether (50:50, v/v) by which the HFB derivatives of PAF, PC, LPC, lyso-PAF, and SM were successfully separated. Their Rf were 0.61, 0.73, 0.47, 0.35, and 0.69, respectively.

Formation of 1-O-Hexadecyl-2-acetyl-*sn*-glycerol (III)

The formation of 1-O-hexadecyl-2-acetyl-*sn*-glycerol (III in Fig. 1) was done according to the method of Satsangi et al,[17] modified as follows. The total amount of compound II is dried down under a stream of nitrogen and 1 mL of acetate buffer pH 4 is added. The hydrolysis is completed after 3 h at 50°C with stirring. The reaction product is extracted twice with 1 mL $CHCl_3$ and the combined chloroform layers are dried under a stream of nitrogen and redissolved in chloroform. The above procedure was also performed on the HFB derivatives of phosphatidylcholine (PC), lysophosphatidylcholine (LPC), lyso-PAF, and sphingomyelin.

A small amount of the hydrolyzed products of PC, LPC, and lyso-PAF was subjected to HPLC analysis, on a silica column, eluted with iso-octane: n-butanol (95:5, v/v). Their retention times, 5.5, 20.0, and 19.0 min, respectively, were identical to the ones of standards distearine, mono-stearine and batyl alcohol, chromatographed under the same conditions. The fraction of the hydrolyzed analog of PC was collected and subjected to ester determination which showed that the yield of the desired product was 76%.

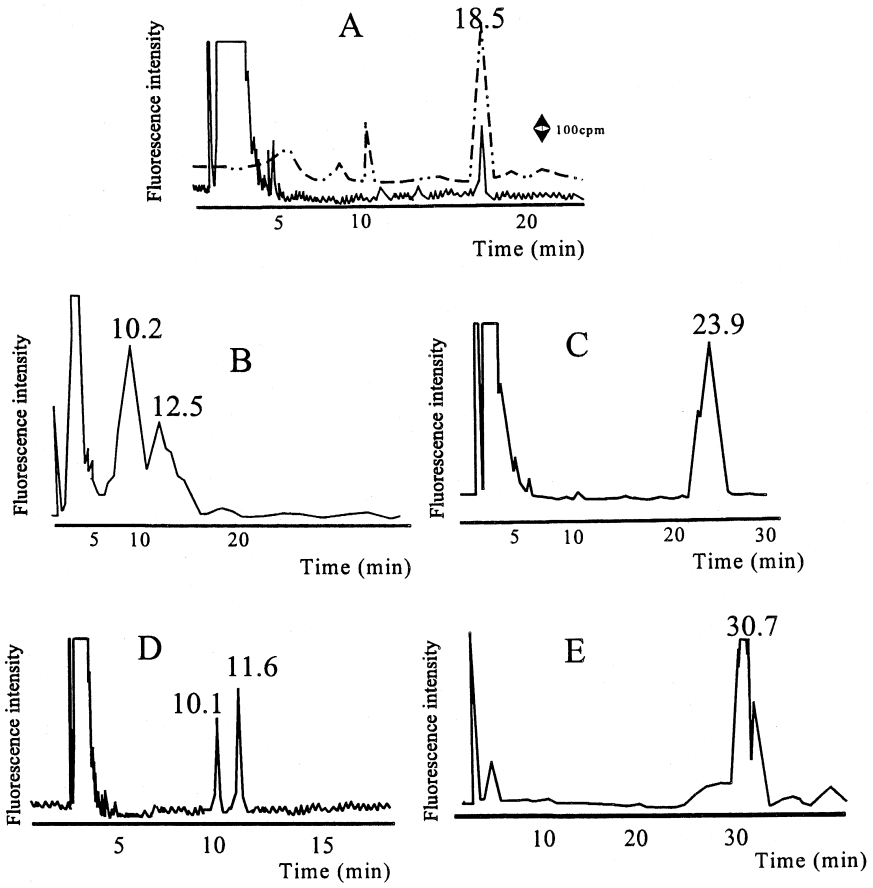

Figure 2. Reverse phase HPLC separations of the methoxycoumarin derivatives of C_{16}-PAF (A), sphingomyelin (B), lyso-PAF (C), lyso-phosphatidylcholine (D) and phosphatidylcholine (E). Reverse phase HPLC separation of the methoxycoumarin derivative of [^{3}H]PAF (----).

A small amount of the hydrolyzed product of PAF was also analyzed under the above chromatographic conditions and a single peak appeared at 10.2 min. This fraction was collected and subjected to ESMS analysis. The ESMS analysis of the compound III showed that an ion, M^+, was present at *m/z* 359 accompanied by an MNa^+ ion at *m/z* 381. Other fragments of interest were: *m/z* 317 $[M\text{-}CH_2CO]^+$ and *m/z* 116 $[M\text{-}C_{16}H_{33}O]^+$.

Preparation of 7-Methoxycoumarin Derivative of the 1-O-Hexadecyl-2-acetyl-*sn*-glycerol (IV)

The preparation of 7-methoxycoumarin derivative of the 1-O-hexadecyl-2-acetyl-*sn*-glycerol (IV in Fig. 1) was performed according to the method of Mita et al.,[18] modified as follows. The compound III is dried under a stream of nitrogen and redissolved in 100 μL 1,2-dimethoxyethane. Then, 10 μg of 7-methoxycoumarin-4-acetic acid and 20 μg of N,N'-dicyclohexylcarbodiimide (DCC) are added. The reaction mixture is kept at 50°C for 3 h. The derivative is extracted twice with 0.5 mL hexane and the extract is transferred to a disposable C_{18} column preconditioned with 10 mL acetonitrile and 5 mL methanol. The column is washed with 6 mL methanol: water (3:1, v/v) followed by 5 mL acetonitrile. The acetonitrile eluate contains the compound IV. The eluate is evaporated to dryness under a stream of nitrogen and redissolved in methanol. The above procedure was also performed on the hydrolysed analogs of phosphatidylcholine (PC), lysophosphatidylcholine (LPC), lyso-PAF, and sphingomyelin (SM).

A small amount of the methoxycoumarin derivatives of PAF, PC, LPC, SM, and lyso-PAF were subjected to HPLC analysis (Fig. 2), on a reverse phase column, eluted with methanol: water (9:1, v/v). Their retention times were: 18.5, 29-32, 10-12, 10-13, and 23.9 min, respectively.

The fraction of the methoxycoumarin derivative of PAF was subjected to ESMS analysis which showed that an ion, M^+, was present at *m/z* 575 accompanied by an ion $[M+ NH_4]^+$, at *m/z* 592. Other fragments of interest were: *m/z* 319, $[M\text{-}CH_2OR]^+$, and *m/z* 225, $[R]^+$.

The overall synthetic procedure was also performed on [^{3}H]PAF (118,000 cpm). The majority of the radioactivity was detected at the peak of the methoxycoumarin derivative of PAF. A small portion of radioactivity (12.7±4.8%, n=4) appeared much earlier which may correspond to underivatized 1-O-hexadecyl-2-acetyl-*sn*-glycerol (Fig. 2). The overall yield of the final product was 49.5±3.7% (n=4), based on the amount of the recovered radioactivity, eluted in the region of the methoxycoumarin derivative of PAF.

Chromatographic Quantitation of PAF

A standard curve of peak area versus concentration of C_{16}-PAF was prepared (Fig. 3). Known amounts of C_{16}-PAF, ranging from 0.5 to 100 ng, were subjected to the entire method in triplicates and the peak areas of the fluorescent derivatives were estimated.

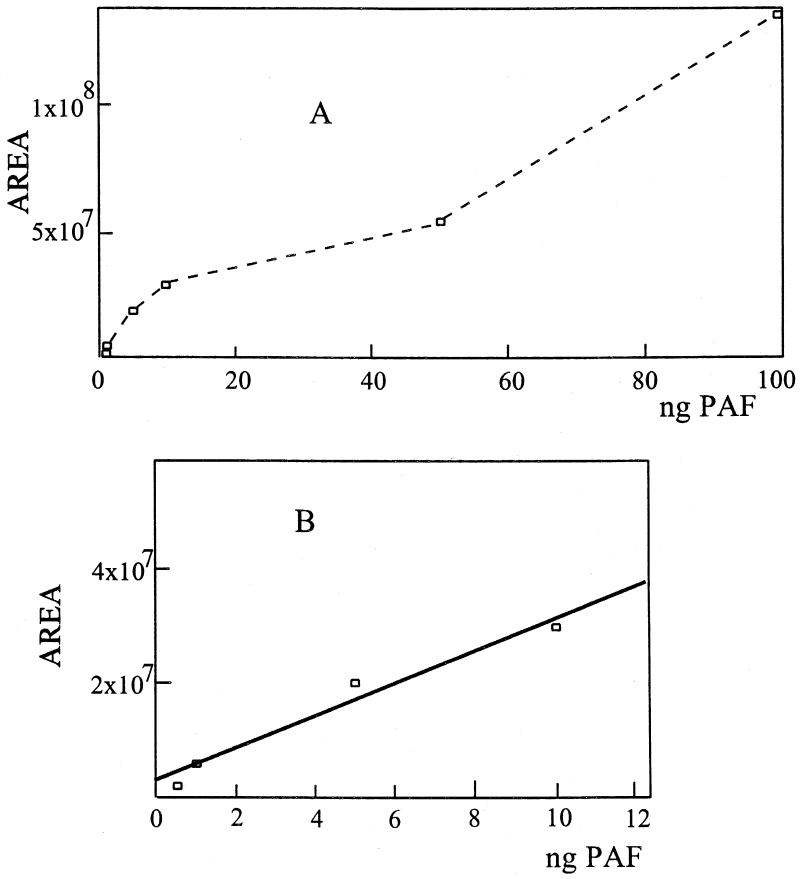

Figure 3. Curve of peak area versus concentration of C_{16}-PAF, ranging from 0.5 to 100 ng (A) and the standard curve of peak area versus concentration of C_{16}-PAF ranging from 0.5 to 10 ng (B).

A linear relationship in the range of 0.5 to 10 ng C_{16}-PAF was observed, with a correlation coefficient 0.984. By analyzing samples of decreasing concentrations, a detection limit of 0.3 ng C_{16}-PAF, with a signal-to-noise ratio 3:1, was obtained.

DISCUSSION

The formation of the 1-O-hexadecyl-2-acetyl-*sn*-glycerol (III) which is subsequently used to synthesize the 7-methoxycoumarin derivative, is generally

prepared by removing the polar head group using phospholipase C. However, several drawbacks have been referred to in this procedure.[17] In order to avoid the enzymatic hydrolysis, we adopted a two-step procedure including synthesis of heptafluorobutyroyl derivative (II) of C_{16}-PAF and formation of 1-O-hexadecyl-2-acetyl-*sn*-glycerol (III). The reaction of the compound II with water was not performed at 35°C overnight, according to Satsangi et al.[17] since our conditions, namely pH 4, at 50°C for 3 h, are not time consuming resulting in increased amount of the final product (IV). The proposed conditions were the optimum ones among others also tested which were different in pH, temperature, and incubation time values.

For the identification of the mono- and diglycerides as well as their glycerylether analogs, resulting from the removal of the polar head group of PC, LPC, lyso-PAF, and PAF, a novel HPLC system was developed. This system also permits a successful separation of glycerides as long as their glycerylether analogs and can be further applied in neutral lipid separation.

A variety of conditions concerning temperature and incubation time values, were tested in the case of 7-methoxycoumarine derivatives preparation in comparison with the ones already published.[18] The superiority of the proposed conditions, namely 50°C for 3 h, lies in the short incubation time and in a higher yield of the fluorescent derivatives. It should be mentioned that a purification procedure is absolutely necessary in this step, in order to separate the fluorescent derivatives from the excess of the fluorescent reagent. The purification method proposed by Mita et al.,[18] was not found appropriate and for this reason, we developed a solid phase separation using C_{18} Sep-Pak cartridges as described in the Results section. However, the purification step can be omitted when the amount of PAF in the initial sample exceeds 1.5 ng. In this case, HPLC analysis of a sample portion up to 20%, leads to sufficient quantification of PAF without any interference of the excess of the fluorescent reagent.

Additionally, the proposed method was applied on standard phospholipids (PC, LPC, SM, and lyso-PAF) whose fluorescent derivatives were completely separated by HPLC from PAF derivative.

In order to evaluate the applicability of the entire method for the determination of PAF in biological fluids, we estimated PAF levels in blood which is considered to be the most complex one. Briefly, 60 mL blood from healthy volunteers were divided in six samples of 10 mL each and the extraction and purification of PAF was made according to the method of Demopoulos et al.[11] In three out of six samples, the estimation of PAF levels was based on the washed rabbit platelet aggregation assay[11] while in the others, the biological assay was substituted by the proposed method.

The PAF levels which were estimated with the proposed method were in accordance with the ones resulting from the biological assay. Although the small number of samples did not permit a statistical analysis, the above results show that the proposed method can be applied in biological fluids.

In conclusion, PAF can be precisely quantitated by the described method with simplicity, sensitivity, without expensive equipment, and can be used in the routine analysis of PAF determination in biological samples and enzyme assays. In addition, the above method also allows the qualitative identification of PAF in contrast to the biological assays which determine PAF-like activity. It can also facilitate qualitative and quantitative identification of polar and neutral lipids, which occur in small amounts in biological tissues and fluids.

ACKNOWLEDGMENT

This work was supported by the General Secretiate for Research and Technology of the Ministry of Development.

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Received April 18, 1998
Accepted May 30, 1998
Manuscript 4765

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