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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF FEMTOMOLAR QUANTITIES OF ENDOGENOUS CARBOXYLIC ACIDS, INCLUDING ARACHIDONIC ACID METABOLITES, AS 4-BROMOMETHYL-7-ACETOXYCOUMARIN DERIVATIVES

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SUMMARY

A major limitation of high-performance liquid chromatographic techniques for measuring biologically active eicosanoids has been the inadequate sensitivity of most on-line detection systems. In addition, the availability of a technique suitable for measuring small quantities of non-esterified fatty acids (NEFAs) in plasma would allow longitudinal studies of plasma levels of these lipids in small animals. To improve the sensitivity of detection, the compounds with acyl groups containing carboxylic acids were derivatized with the highly fluorescent compound, 4-bromomethyl-7-acetoxycoumarin. All classes of NEFA and arachidonic acid metabolites, including the cyclooxygenase and lipoxygenase products, and hydroxy acid compounds could be derivatized with this reagent. The derivatized metabolites were separated with a reversed-phase high-performance liquid chromatographic system using a radial compression column and a gradient elution technique. Reproducible measurements of plasma NEFAs from as little as 5 μl of plasma, and femtomolar concentrations of eicosanoids, could be detected using an on-line fluorescent spectrometer. This improvement in sensitivity should permit the quantification of all eicosanoids, including the leukotrienes, in biologic fluids and the longitudinal measurement of changes in plasma NEFA levels in small animals.

INTRODUCTION

Although high-performance liquid chromatography (HPLC) is routinely used to separate arachidonate metabolites, a major disadvantage has been the inadequate sensitivity of on-line detection techniques [1]. This has necessitated the use of a separate assay technique, such as mass spectroscopy, radioimmunoassay

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or bioassay. If a highly fluorescent compound capable of derivatizing all eicosanoids were available, an on-line fluorescence detector could be used to avoid the shortcomings of available techniques.

Recently, Tsuchiya et al. [2] reported an HPLC technique for the detection of carboxylic acids following precolumn derivatization with 4-bromomethyl-7-acetoxycoumarin (Br-Mac). They subsequently reported that this technique could be used to accurately measure cyclooxygenase metabolites of arachidonic acid and plasma non-esterified fatty acids (NEFAs) [3,4]. The ability of this technique to detect other classes of arachidonic acid metabolites was not reported. A major advantage of this coumarin compound over the more commonly used 4-bromomethyl-7-methoxycoumarin (Br-Mmc) is that its fluorescence intensity does not change appreciably with variations in the composition of the mobile phase. Consequently, gradient-elution HPLC techniques can be used. Coumarin derivatives with an acetoxy group in the 7-position are more fluorescent than 7-methoxy derivatives, but only in an alkaline solution [5]. To take advantage of this property, the technique of Tsuchiya et al. [2] requires on-line post-column alkaline hydrolysis of derivatized carboxylic acids.

In this work, we report that Br-Mac can be used to derivatize all classes of eicosanoids, including the hydroxy acid (hydroxyeicosatetraenoic acid, HETE) metabolites and the leukotrienes. In addition, we found that a radial compression C_{18} column resulted in more rapid separations of eicosanoids than could be achieved with stainless-steel columns. The technique developed by Tsuchiya et al. [4] was also easily adapted to serial measurements of plasma NEFAs in conscious, restrained rats.

EXPERIMENTAL

Materials

All analytical-grade reagents used to synthesize Br-Mac were obtained from Aldrich (Milwaukee, WI, U.S.A.), with the exception of α,α-azobisisobutyronitrile, which was obtained from Fluka (Houppauge, NY, U.S.A.). Chromatography solvents were HPLC grade and were obtained from Fisher Scientific (Medford, MA, U.S.A.). Eicosanoids and fatty acid standards were obtained from either Sigma (St. Louis, MO, U.S.A.) or Cayman Biologics (Ann Arbor, MI, U.S.A.).

High-performance liquid chromatography

The Waters (Milford, MA, U.S.A.) HPLC equipment we used is illustrated schematically in Fig. 1, and consisted of a WISP Model 710B automatic injector, two 510A HPLC pumps, a Model 480 fluorescence detector (excitation filter 365 nm, emission filter 460 nm), a 720 system controller, and a data module peak integrator. The columns obtained from Waters were: a radial compression C_{18} reversed-phase column (Nova Pak, 5 μm particle size, 10 cm$\times$5 mm), two C_{18} columns (μBondapak, 10 μm particle size, 30 cm$\times$3.9 mm, and Resolve, 5 μm particle size, 15 cm$\times$3.9 mm) and a phenylpropylsilane reversed-phase column (fatty acid analysis column, 10 μm particle size, 30 cm$\times$3.9 mm). A third, low-

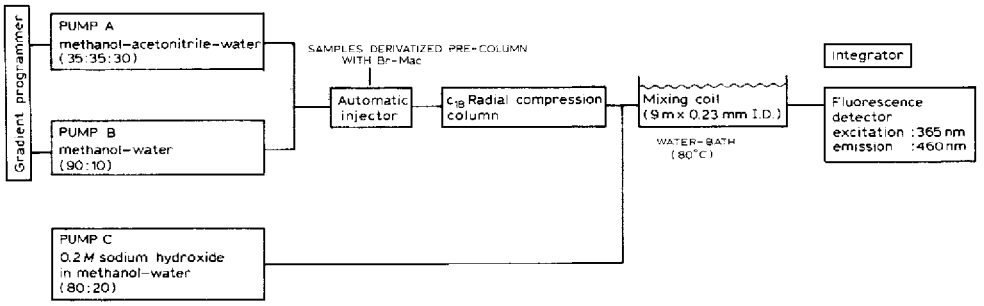


Fig. 1. HPLC system for quantification of Br-Mac derivatives of arachidonic acid metabolites.

pressure pump (Instrument MiniPump, Milton Roy, St. Petersburg, FL, U.S.A.) was used to mix the alkaline hydrolysis solution with the Br-Mac derivatives following their separation on the HPLC system. The mixing coil was constructed by joining three pieces of 3 m×0.23 mm I.D. stainless-steel tubing. The mixing coil temperature was controlled using a water bath.

Synthesis of Br-Mac

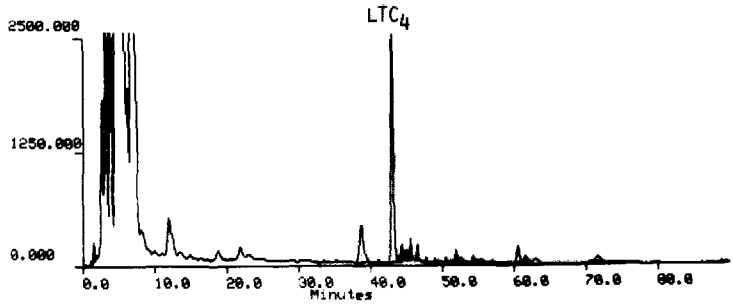
Br-Mac is not available commercially; therefore, we followed the procedures of Tsuchiya et al. [2] to synthesize this compound. Briefly, 4-methyl-7-hydroxycoumarin (50 g) and acetic anhydride (100 ml) were refluxed for 1 h and then poured into 500 ml of cold water. The resulting solid product, 4-methyl-7-acetoxycoumarin, was filtered and recrystallized from ethanol. Approximately 10 g of this compound were refluxed for 20 h with 9 g of N-bromosuccinimide and 0.6 g of the catalyst, α,α-azobisisobutyronitrile, in 100 ml of carbon tetrachloride. After removal of carbon tetrachloride by evaporation, the residue was washed with water on a sintered-glass filter and recrystallized first from ethyl acetate and then from cyclohexane. The recrystallization steps were repeated and produced a compound with a melting point of 181–182°C. The yield was approximately 1 g, an amount sufficient for approximately 20 000 assays. The compound does not lose activity for over six months if kept desiccated at −20°C.

Plasma NEFAs

Blood was obtained from the tail vein of Sprague–Dawley rats weighing 100–400 g on a variety of experimental protocols. The animals, which had been trained to enter a restraining cage used for determination of blood pressure using the indirect tail cuff technique, were allowed to remain in the cage for 1 min with the tail immersed in water at 37°C. The tip (2–3 mm) of the tail was removed with a scalpel, and 50–75 μl of blood were allowed to collect in a heparinized glass capillary tube. The blood was centrifuged at 2000 *g* and plasma samples were frozen overnight. Serial tail vein blood samplings could be obtained in this manner with no wound infections in any animals.

Determination of plasma NEFAs

Plasma samples were never left frozen for more than 18 h before extraction. To 15-μl aliquots of plasma, 3 nmol of $C_{17:0}$ (heptadecanoic acid; Sigma) in water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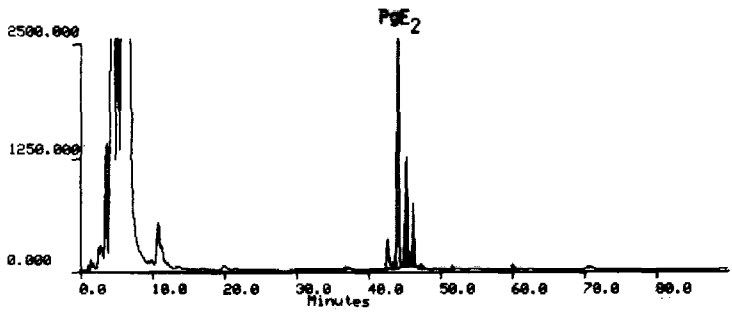
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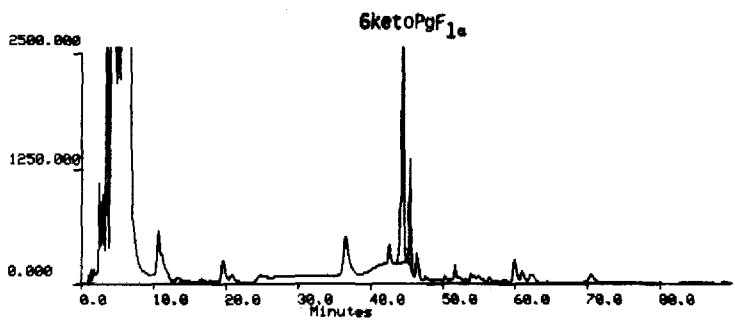
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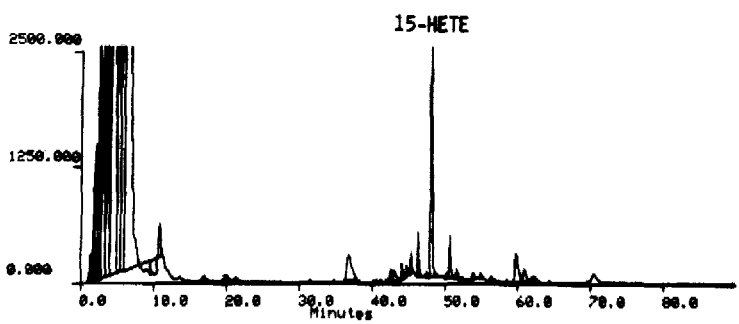
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were added as an internal standard (final volume of 500 μl). Methanol (1 ml) was added and the samples were vortexed. Chloroform (3 ml) containing 5 mg per 100 ml butylated hydroxytoluene (BHT) were added and the samples vortexed for 1 min and centrifuged at 2000 *g* for 10 min. The organic layer was removed and the aqueous layer was washed once in 4 ml of chloroform–methanol (3:1, v/v). The pooled organic phases were dried under nitrogen, reconstituted in 1 ml of chloroform, and applied to aminopropyl bonded-phase disposable columns (Bond Elut, Analytichem International, Harbor City, CA, U.S.A.) to separate NEFAs from neutral lipid and phospholipid, according to the technique of Kaluzny et al. [6]. Fractions containing NEFAs were eluted with 2% acetic acid in diethyl ether, dried under nitrogen and reconstituted in acetone. The recovery of saturated and unsaturated NEFAs was $\geqslant$90%, as determined by the recovery of radiolabelled NEFA standards.

Derivatization technique

The derivatization of each eicosanoid or the NEFA plasma fractions was carried out using the procedure for carboxylic acids, as detailed by Tsuchiya et al. [2]. Approximately 10 mg of Br-Mac stock were dissolved in 10 ml of acetone (approximately 2 μmol/ml); this solution was stable for three days at 4 °C in the dark. A 50-μl aliquot of the Br-Mac stock solution was added to 50 μl of a dibenzo-18-crown-6 acetone solution (40 nmol per 50 μl) in a 1-ml glass ampoule. The eicosanoid standards (100 fmol–1 nmol) were added with 2–3 mg of a finely powdered mixture of potassium bicarbonate and sodium sulfate. The ampoule was sealed, covered with aluminum foil and incubated at 50 °C for 30 min in a shaking water bath. A 5–50 μl aliquot of this mixture was injected onto the column. One technician was capable of derivatizing up to 30 samples an hour using this technique.

RESULTS AND DISCUSSION

Initially, we used several reversed-phase stainless-steel columns and varied column temperatures between 22 and 50 °C. Although adequate resolution of all fatty acid standards, including HETE metabolites of arachidonic acid, was achieved with the 5-μm C_{18} column, the analysis times usually exceeded 90 min.

Fig. 2. Chromatographic separations of several of the major classes of eicosanoids are illustrated. The retention times of thromboxane B_2, prostaglandins B, D_2 and E_1 and other HETE compounds varied from 38 to 50 min (not illustrated). Arachidonic acid eluted at 54 min using this gradient elution scheme. The initial mobile phase was acetonitrile–methanol–water (35:35:30, v/v/v) (solvent A); solvent B was methanol–water (90:10, v/v). A convex gradient to 50% solvent B over 40 min was used, followed by a shallow linear gradient to 100% solvent B over 40 min. The mobile phase flow-rate in all examples illustrated was 1.5 ml/min; the flow-rate of the alkaline hydrolysis solution was 0.4 ml/min. Approximately 100 pmol of each standard were injected. The initial large unlabelled peaks in each chromatogram represent the elution of underivatized Br-Mac; no other peaks were detected in the solvent blank. The smaller peaks detected preceding and following each standard represent impurities contained with the eicosanoid standard or a metabolite. Peaks: LTC_4 = leukotriene C_4; PgE_2 = prostaglandin E_2; 6ketoPg$F_{1\alpha}$ = 6-keto-prostaglandin $F_{1\alpha}$; 15-HETE = 15-hydroxyeicosatetraenoic acid.

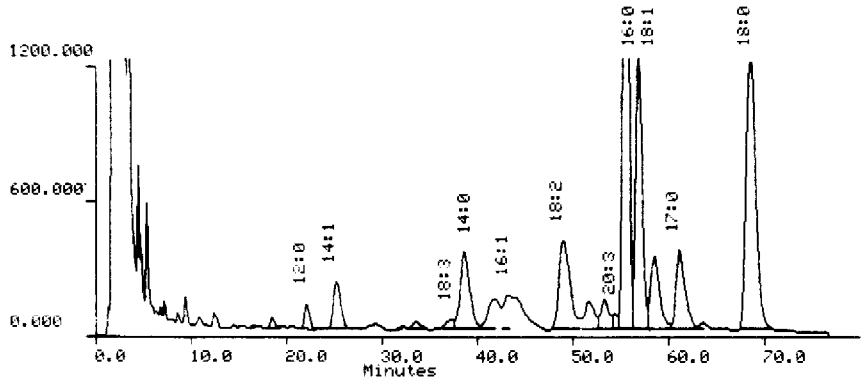

Fig. 3. Typical chromatogram of plasma NEFAs derivatized with Br-Mac and eluted over 70 min with a convex gradient of solvent A (acetonitrile–methanol–water, 35:35:30, v/v/v) to solvent B (methanol–water, 90:10, v/v) at a flow-rate of 2 ml/min; the alkaline hydrolysis solvent flow-rate was 0.4 ml/min. The chromatogram shown represents NEFAs in 10 μl of rat plasma; 17:0 is the internal standard; 20:4, not labelled, is the peak immediately preceding 16:1. These separations were achieved with a Waters Nova Pak 4-μm radial compression column. The reagent blank contained only the underivatized Br-Mac reagent that eluted typically in less than 10 min; no other peaks were detected. All NEFA standards were equally stable during the derivatization technique (recoveries of >90%) and in storage in chloroform with 5 mg% BHT at $-10°C$ for one month.

Increasing the column temperature, as discussed by Tsuchiya et al. [4], decreased retention times but our ability to resolve closely related compounds declined, particularly the HETE derivatives and 16:1 and 20:4 NEFA standards. We also tested a radial compression, reversed-phase column which has recently been shown to separate underivatized arachidonic acid metabolites [7]. Using a gradient elution technique which varied the ratio of acetonitrile to methanol, we found that the C_{18} radial compression column was capable of resolving all free fatty acid and eicosanoid standards tested at room temperature. These separations required less than 70 min. This technique allowed us to take advantage of the increased flexibility and reduced cost of radial compression columns while avoiding the requirement for a column heater.

The important advantage of the Br-Mac technique is the intense fluorescence of 7-acetoxycoumarin compounds in alkaline solutions [2]. Therefore, an additional low-pressure pump was necessary for the post-column hydrolysis of Br-Mac from each derivatized carboxylic acid. We constructed a mixing coil using 9 m of stainless-steel tubing with a very narrow I.D. (0.23 mm) that allowed complete hydrolysis to occur while minimizing band spreading. We confirmed that the sensitivity of detection can be enhanced by increasing the temperature of the mixing coil to about 80°C; any further increase in temperature did not result in an appreciable increase in sensitivity. In Fig. 2, the separation and detection of eicosanoid standards using several gradient elution programs are shown. For the cyclooxygenase metabolites, those with increasing unsaturation of the acyl chain and/or increasing numbers of hydroxyl groups on the cyclopentane ring tended to have shorter retention times. In Fig. 3, the results of a typical chromatographic separation of NEFAs are illustrated; 5 μl of plasma were required for reproducible

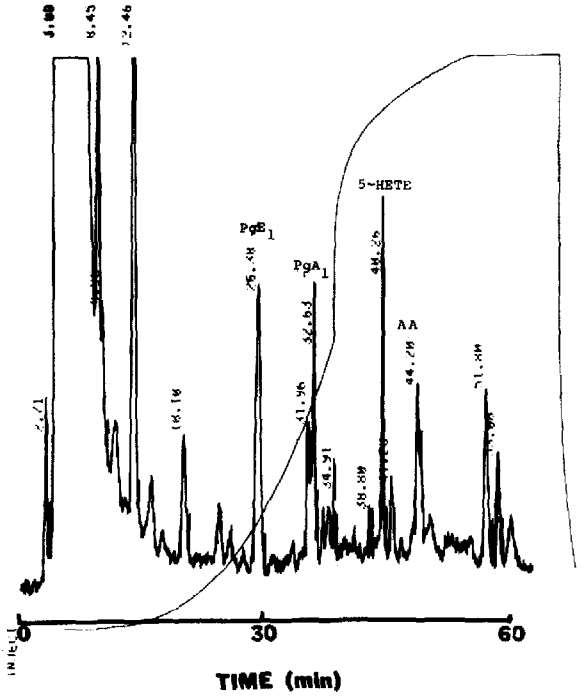

Fig. 4. A 50-fmol amount of each of several eicosanoid standards was derivatized with Br-Mac, as described in Experimental, and injected onto the radial compression column. The initial mobile phase was acetonitrile–methanol–water (35:35:30, v/v/v); a second tracing illustrates the changing ratio of solvent A to solvent B (methanol–water, 90:10, v/v) from 0% B to 100% B. The solvent flow-rate was 1.0 ml/min. The gain setting on the Waters Model 480 fluorescence detector was ×64. A reagent blank at this level of sensitivity contained the usual initial underivatized Br-Mac peak, and a number of smaller, unidentified peaks that represent contaminating compounds with free carbocyclic groups. Nevertheless, each of the added eicosanoid standards could be quantified at this concentration, although the coefficient of variation from analysis to analysis was high (10–20%). Peaks: PgE_1 = prostaglandin E_1; PGA_1 = prostaglandin A_1; 5-HETE = 5-hydroxyeicosatetraenoic acid; AA = arachidonic acid.

results using $C_{17:0}$ as an internal standard. Increasing acyl chain length resulted in longer retention times while increasing number of double bonds (all *cis*) shortened retention times in this reversed-phase system. The within-day coefficient of variation was less than 2%, and a day-to-day variation of less than 5% was noted with at least 7 pmol of each NEFA injected. The detection limit was 10 fmol using the Waters Model 480 fluorometer, although the assay-to-assay variability was increased at this sensitivity (10–20%).

The separation of several eicosanoid standards contained in a single injection is shown in Fig. 4. A 50-fmol amount of each compound was clearly resolved. Since eicosanoids in physiological fluids are present in the pg/ml range [1], this method should be sufficient to quantify biologically relevant concentrations of arachidonic acid metabolites. Although the recovery of LTC_4 was lower than that of other eicosanoids tested, recent reports suggest methods that could improve the recovery of the peptidoleukotrienes. For example, Henke et al. [7] noted that the recovery of peptidoleukotrienes improved when columns were pretreated with

EDTA. Powell [8] reported that adding relatively high concentrations (0.5–2.5 m*M*) of trifluoroacetic acid to acetonitrile–methanol gradient elutions improved the resolution of lipoxygenase products without affecting the retention times of other arachidonate metabolites [8].

With the Br-Mac method, it should be possible for the first time to quantify arachidonic acid metabolites, including the lipoxygenase products, in biologic fluids. The synthesis of Br-Mac is straightforward, with a high yield of the compound, and the derivatization step is rapid and complete. The technique takes advantage of the high resolution of HPLC and has a sensitivity comparable to either mass spectrometry or radioimmunoassay. Other single-step HPLC methods that have been reported do not have the sensitivity required to detect eicosanoids in biologic fluids [9], or they require complex column-switching devices [10]. McGuffin and Zare [11] recently reported the successful resolution of femtomolar quantities of eicosanoids using the Br-Mmc derivative, although this technique employed a laser fluorescence detector. The Br-Mac method allows the experimenter to identify and quantify arachidonate metabolites or small amounts of individual free fatty acids in a single chromatographic determination using standard HPLC laboratory equipment.

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