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Measurement of 5,8,11,14-eicosatetraynoic acid in plasma by gas–liquid chromatography

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Summary A gas–liquid chromatography–flame ionization detection method is described for measuring plasma levels of eicosatetraynoic acid using eicosatriynoic acid as an internal standard. The technique is simple, rapid, and reproducible. Eicosatetraynoic acid and eicosatriynoic acid behave similarly in the extraction system chosen, yet can be easily resolved by gas–liquid chromatography. Under the conditions of our assay, most plasma fatty acids have a short retention time and are found near the solvent front, whereas, eicosatetraynoic acid and eicosatriynoic acid are retained for 6.9 and 3.75 minutes, respectively.—**Goodman, R. P., and A. R. Brash.** Measurement of 5,8,11,14-eicosatetraynoic acid in plasma by gas–liquid chromatography. *J. Lipid Res.* 1981. **22:** 541–543.

Supplementary key word eicosatriynoic acid

Eicosatetraynoic acid (ETYA) is the acetylenic analogue of arachidonic acid. In vitro, ETYA is both a cyclooxygenase and lipoxygenase inhibitor with slightly greater potency for the lipoxygenase pathway (1, 2). Accordingly, it has potential value as a research tool for assessing the role of the lipoxygenase pathway in physiologic and pathologic states in vivo in experimental animals and in man. Interpretation of such studies requires assurance that ETYA is present in concentrations sufficient to exert an inhibitory effect. With this in mind, we have developed a simple assay to measure plasma levels of ETYA using eicosatriynoic acid (ETRIYA) as an internal standard.

Procedure and results

Stock solutions of ETRIYA, 8,11,14-eicosatriynoic acid, and ETYA 5,8,11,14-eicosatetraynoic acid (gifts

Abbreviations: GLC–FID, gas–liquid chromatography–flame ionization detection; ETYA, 5,8,11,14-eicosatetraynoic acid; ETRIYA, 8,11,14-eicosatriynoic acid.

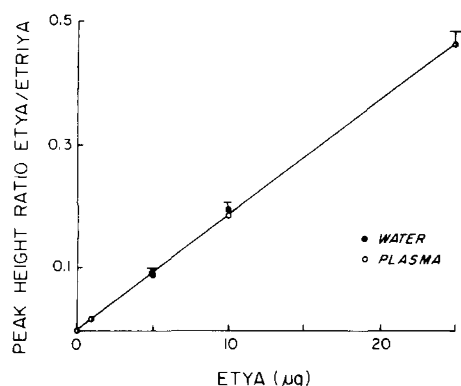

Fig. 1. Standard curve derived from various amounts of ETYA (values on abscissa) and ETRIYA (20 μg) in 1 ml of plasma (○) or water (●). The ratios are linear over the range of the standard curve, and the extraction ratios from plasma and water are identical (n = 3).

from Dr. James Hamilton, Hoffman-La Roche, Inc., Nutley, NJ), were prepared by dissolving the compounds in methanol to a final concentration of 1 μg per μl. The solutions were stored under nitrogen in the dark at −20°C. The stock solutions were stable for at least 5 months as determined by examination on several different GLC phases. Blood samples were collected in tubes containing heparin (Venoject KT200KA; Elkton, MD) and centrifuged to separate the plasma. Twenty microliters (20 μg) of the ETRIYA stock solution was added to 1 ml of plasma as the internal standard. The plasma was then assayed immediately or after storage for up to 1 week at −20°C. The plasma was mixed with 5 ml of isopropyl alcohol–n-heptane–1 N sulfuric acid 40:10:1 and left at room temperature for 10 min. To this solution was then added 2 ml of heptane and 3 ml of distilled water. After vigorous shaking, the layers were allowed to separate. The yellow upper organic phase (approximately 3 ml) was pipetted off and evaporated to dryness under nitrogen. The residue was then dissolved in 100 μl of methanol and 1 ml of an ethereal solution of diazomethane and left at room temperature for 10 min. The sample was reevaporated under nitrogen and dissolved in 30 μl of hexane. One microliter of this solution was injected for quantification. The GLC–FID apparatus used a silanized glass column (6 ft × ¼″) packed with 3% SP-2310 on 100/120 Supelcoport (Supelco, Inc., Bellefonte, PA). The oven was maintained isothermically at a temperature of 225°C. Nitrogen at a flow rate of 40 ml/min was employed as a carrier gas. The retention time for the methyl ester of ETYA was 6.9 min and for the methyl ester of ETRIYA was 3.75 min. A five-point standard curve was constructed using peak height ratios of ETYA to ETRIYA; various amounts of the ETYA stock solution and 20 μg of the ETRIYA stock solution were added to 1-ml aliquots of plasma, extracted, and analyzed by GLC-FID. The standard curve was linear (**Fig. 1**). The concentration of ETYA in plasma samples was estimated by fitting the peak height ratios of the fatty acids on the standard curve. Using this technique, the coefficient of variation at 1 μg per ml of ETYA (our lower limit of accepted concentration) within assay was 6% and between assay was 13% (n = 5). The coefficient of variation at 10 μg per ml of ETYA for different plasma samples (n = 5) was 5%. The recovery of ETYA was estimated by adding ETYA (10 μg) to 1 ml of plasma before extraction and ETRIYA (20 μg) after extraction and comparing the ratio with a pure mixture of the two fatty acids. The recovery of ETYA was determined to be 69% ± 4% (n = 3) and using a similar procedure for ETRIYA, was 63% ± 2% (n = 3). Preliminary studies in four volunteers indicate that maximum plasma concentrations in the range of 3.1 to 9.8 μg per ml can be achieved after a single oral dose of 500 mg and can be readily quantified by this assay.

Comments

Our method of analysis is simple, rapid, and reproducible. The addition of an internal standard to the plasma followed by protein denaturation, extraction, esterification, and then analysis by GLC–FID is certainly not a novel approach and has been used for many other fatty acids including arachidonic acid (3). The physiochemical properties of ETYA, however,

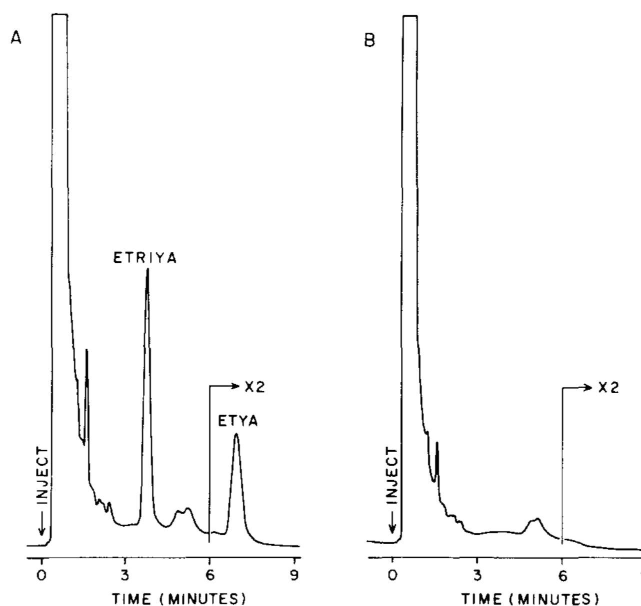

Fig. 2. GLC-FID profiles of plasma containing 10 μg per ml ETYA and 20 μg per ml ETRIYA (A) and blank plasma (B). ETYA and the internal standard ETRIYA are well resolved from interfering substances in plasma.

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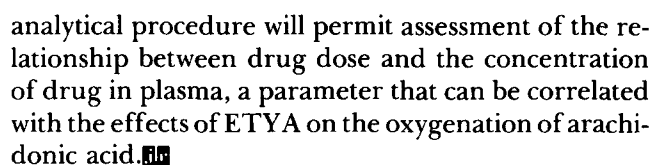
differ from those of other unsaturated fatty acids because of the four acetylenic bonds and make this a more complicated problem. We initially tried several combinations of organic solvents for extraction of both neutral and acidified plasma, but obtained either very poor recovery or an extract which would have required an additional purification procedure. We finally chose a method based on that developed by Dole (4) for the measurement of non-esterified fatty acids in plasma. Although Dole (4) and Goss and Lein (5) using this method report extraction of unsaturated fatty acids to approach 100%, we find that the extraction of ETYA is less complete. The choice of an internal standard with three acetylenic bonds and extraction properties similar to ETYA compensates for the lower recovery. Attempts to use the saturated fatty acids stearic acid and lignoceric acid or the unsaturated fatty acid dihomo-γ-linolenic acid as internal standards did not give reproducible ratios. The extraction method is not selective so that there is the potential for many contaminating peaks on GLC–FID. The selection of a relatively polar phase insures that nearly all the extracted compounds chromatograph near the solvent front while ETYA and ETRIYA possess longer retention times and can be adequately separated (**Fig. 2**). This analytical procedure will permit assessment of the relationship between drug dose and the concentration of drug in plasma, a parameter that can be correlated with the effects of ETYA on the oxygenation of arachidonic acid.



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