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SCIENCE DIRECT

Vascular Pharmacology 42 (2005) 163–169

Vascular Pharmacology

www.elsevier.com/locate/vph

Rapid and simple determination of epoxyeicosatrienoic acids in rabbit renal artery by reversed-phase HPLC with fluorescence detection

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Received 13 July 2004; accepted 20 December 2004

Abstract

A liquid chromatographic method with fluorescence detection coupled with a solid-phase extraction was applied to the rapid determination of epoxyeicosatrienoic acids (EETs) in the rabbit renal artery. The EETs were extracted with an acetonitrile from renal artery homogenate and concentrated by a solid-phase extraction method. The concentrated EETs were reacted directly with a 6, 7-dimethoxy-1-methyl-2 (1*H*)-quinoxalinone-3-propionyl-carboxylic acid (DMEQ) hydrazide and separated by a reversed-phase HPLC with eluting a combination of a step-wise and a gradient of a mixture of methanol and water. The content of EETs in the renal arteries was significantly greater in the 0.5% cholesterol fed rabbits than in control rabbits. It is suggested that hyperchlesterolemia increases the production of EETs in the rabbit renal artery.

Keywords: EDHF; Epoxyeicosatrienoic acids (EETs); Rabbit renal artery; Reversed phase liquid chromatography; Fluorescence detection

1. Introduction

Cytochrome *P*-450 converts arachidonic acid (AA) to 4 regioisometric epoxyeicosatrienoic acids (EETs): 5, 6-, 8, 9-, 11, 12- and 14, 15-EET (Capdevila et al., 1992; McGiff, 1991). EETs are produced by vascular endothelium and are considered important regulators of vascular tone (Harder et al., 1995; Rosolowsky and Campbell, 1993, 1996). EETs induce the relaxation in the cerebral, coronary and renal circulation (Ellis et al., 1990; Katoh et al., 1991; Oyekan et al., 1994), and this vasorelaxation has mainly been attributed to hyperpolarization in the vascular smooth muscle cell through the activation of calcium-activated potassium channels (Campbell et al., 1996; Gebremehin et al., 1992). Recently, many studies have suggested that EETs are one of the candidates of endothelium-derived hyperpolarizing factor (EDHF), an unidentified relaxant produced by endothelium (Campbell et al., 1996; Bauersachs et al., 1994; Fulton et al., 1995; Hecker et al., 1994; Honda et al., 2001a,b; Iwata and Honda, 2004).

It has been indicated that all 4 regioisometric EETs, 5, 6-, 8, 9-, 11, 12- and 14, 15-EET, relax canine and bovine coronary arteries equally (Rosolowsky and Campbell, 1993, 1996; Campbell et al., 1996). In contrast, 5, 6-EET was more active than the other regioisometric EETs in relaxing rat and rabbit mesenteric arteries, pig and rabbit cerebral arteries, and rat tail artery (Ellis et al., 1990; Huntcheson et al., 1999; Carroll et al., 1987; Leffer

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doi:10.1016/j.vph.2004.12.003

and Fedinec, 1997; Proctor et al., 1987). Further, recent study has indicated that 5, 6-EET produces dose-dependent and potent relaxation in the rabbit arteries (Taylor et al., 2001).

Although an importance of EETs in a variety of physiological and pharmacological actions, there are few methods developed to determine EETs in biological samples. The most common method for determining EETs has been the method using radioisotope (Laethem et al., 1992), photodiode array detector (Kiss et al., 1998) and a microbore column (Nithipatikom et al., 2000). To our knowledge, however, no report has yet been published describing the simple and rapid determination of EETs in a biological sample.

In this study, we report here a simple and a rapid determination method of EETs in a rabbit renal artery by off-line solid-phase extraction followed by a reversed-phase HPLC with fluorescence detection. This assay reaches the required level of sensitivity and reproducibility for determining EETs in the rabbit renal artery.

2. Materials and methods

2.1. Animals and tissues

This investigation conforms with the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). Ten-week-old male Jla:JW rabbits supplied by Japan Laboratory Animal (Tokyo, Japan) were used. Rabbits were fed normal chow, 0.5% cholesterol chow for 5 weeks. The animals were anesthetized with pentobarbital (40 mg/kg, iv) and sacrificed by bleeding. The renal artery was isolated and placed in modified ice cold Krebs–Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25.0 mM $NaHCO_3$ and11.0 mM glucose) gassed with 95% O_2 and 5% CO_2 (Honda et al., 1999; Ampong et al., 2002). The tissues were cleaned by removing connective tissue and then quick-frozen in liquid nitrogen.

2.2. Sample preparation

The rabbit renal artery was homogenated with 1 ml volume of Tris buffered saline (137 mM NaCl, 2.7 mM KCl, 25 mM Tris, 0.1% Tween 20, pH 7.4) using Ultra-Turrax homogenizer (IKA-WERKE, Staufen, Germany) under cooling with ice. Then, a 3 ml-volume of acetonitrile was added to the homogenate and vigorously mixed for 10 min to extract the 5, 6-EET. The extract was centrifuged at 3000 rpm for 10 min at 4 °C using KOKUSAN H-501FR (KOKUSAN, Tokyo, Japan). After the supernatant was filtered through a MILIPORE filter (0.45 μm) (Millex, Millipore, Bedford, MA, USA), a 3 ml-volume of water was added to the filtrate and mixed well. The filtrate was applied to the conditioned extraction column Oasis HLB cartridge (Waters, MA, USA) at a flow-rate of one drop/s, then 5, 6-EET was completely adsorbed on the extraction cartridges. The adsorbed 5, 6-EET was recovered from the cartridges by eluting with a 100 μl-volume of pure acetonitrile. After the solvent was dried under reduced pressure and 1 ml-volume of water was added, then, the mixture was lyophilized by EYELA FD-1000 (Tokyo Rikakikai, Tokyo, Japan).

2.3. Fluorescent derivatization

The 5, 6-EET standards or samples (extracted from a rabbit renal artery) were dissolved in 10 μl-volume of *N*, *N*-dimethylformamide. Five microliter-volume of 2 M EDC aqueous solution, 5 μl 10% pyridine and 10 μl 4.9 mM DMEQ-hydrazide in a *N*, *N*-dimethylformamide solution were added to the solution successively. The mixtures were vigorously stirred for 15 min. The carboxyl group of the EET was easily reacted with DMEQ-hydrazide to produce a strong fluorescent derivative as shown in Fig. 1. The derivative was stable for 2 h at room temperature.

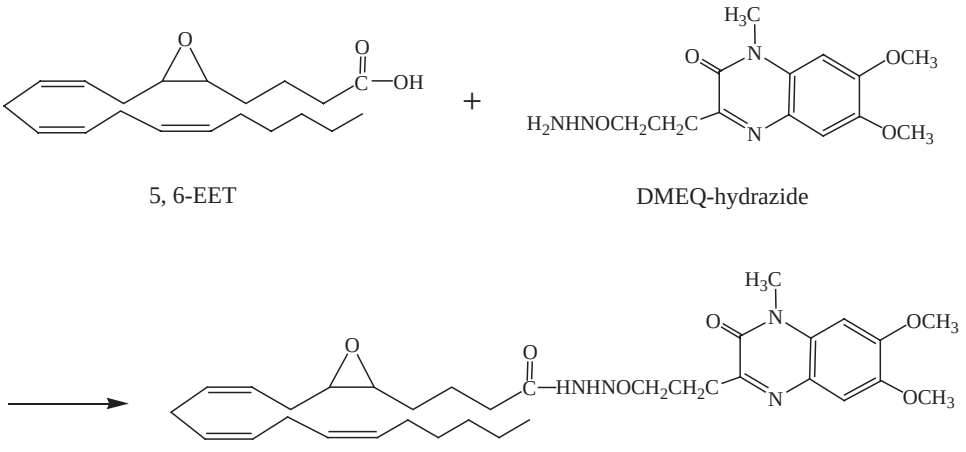

Fig. 1. Fluorescence labeling of 5, 6-EET with DMEQ-hydrazide.