Free Radical Biology and Medicine 99 (2016) 128–138

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

A comprehensive evaluation of the [2-^{14}C](–)-epicatechin metabolome in rats

Gina Borges, Justin J.J. van der Hooft [1], Alan Crozier *

School of Medicine, Dentistry and Nursing, College of Medical, Veterinary and Life Science, University of Glasgow, Glasgow, UK

ARTICLE INFO

Article history:
Received 17 June 2016
Received in revised form
26 July 2016
Accepted 1 August 2016
Available online 3 August 2016

Keywords:
[2-^{14}C](–)-Epicatechin
Rats
Acute ingestion
Absorption
Disposition
Metabolism
Excretion

ABSTRACT

Following ingestion of [2-^{14}C](–)-epicatechin by rats, radioactivity in urine, feces, body fluids and tissues collected over a 72 h period, was measured and ^{14}C-metabolites were analyzed by HPLC-MS^2 with a radioactivity monitor. In total 78% of the ingested radioactivity was absorbed from the gastrointestinal tract (GIT), and then rapidly eliminated from the circulatory system via renal excretion. A peak plasma concentration occurred 1 h after intake corresponding to ~0.7% of intake. Low amounts of radioactivity, < 2% of intake, appeared transiently in body tissues. Glucuronidation and methylation of (–)-epicatechin began in the duodenum but occurred more extensively in the jejunum/ileum. Radioactivity reaching the cecum after 6–12 h was predominantly in the form of the ring fission metabolites 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 5-(3′,4′-dihydroxyphenyl)-γ-hydroxyvaleric acid along with smaller amounts of their phase II metabolites. Low levels of metabolites were detected in the colon. Of the ingested radioactivity, 19% was voided in feces principally as ring-fission metabolites. The main components in plasma were (–)-epicatechin-5-*O*-glucuronide and 3′-*O*-methyl-(–)-epicatechin-5-*O*-glucuronide with small amounts of (–)-epicatechin, 3′-*O*-methyl-(–)-epicatechin, 5-(3′-hydroxyphenyl)-γ-hydroxyvaleric acid-4′-glucuronide and hippuric acid also being detected. No oxidized products of (–)-epicatechin were detected. No compelling evidence was obtained for biliary recycling of metabolites. The findings demonstrate substantial differences in the metabolism of (–)-epicatechin by rats and humans. Caution should, therefore, be exercised when using animal models to draw conclusions about effects induced by (–)-epicatechin intake in humans.

1. Introduction

Flavan-3-ols, both as monomers, such as (–)-epicatechin, and proanthocyanidins with varying degrees of polymerization, are widely distributed in the diet, including apples and berries, with especially high levels being found in green tea and cocoa and some of their derived products [1–3]. Flavan-3-ols have been implicated in a variety of protective effects on human health [4,5] with dietary intervention studies having shown improved vascular function [6,7] as well as beneficial effects on cognition [8,9]. The molecular events and mechanism behind these effects remain elusive. There is, however, a growing realization that information on the absorption, distribution, metabolism and excretion (ADME) (aka bioavailability) of flavan-3-ols following ingestion is essential if the mode of action underlying their beneficial effects is to be elucidated [10–12].

While there is an increasing amount of information on the ADME of (–)-epicatechin in humans [12–14], limited consideration has been given to the importance of the (–)-epicatechin ADME in animal models given the available evidence indicates that the circulating profile of (–)-epicatechin metabolites in humans is different from that of rats, but has some similarities to that of mice [12,15]. Little is known about other aspects of animal and human ADME processes: in particular potential differences in the profile of gut microbiome-derived (–)-epicatechin derivatives, including ring fission metabolites such as hydroxyphenyl-γ-valerolactones and valeric acids. An appreciation and a more detailed characterization of the ADME of (–)-epicatechin in animal models is of particular importance in mechanistic studies with in vitro and ex vivo test systems.

This paper reports on a study on the ADME of [2-^{14}C]

Abbreviations: MS, mass spectrometry; RC, radiocounting; SREM, structurally-related epicatechin metabolite; 5C-RFM, ring fission metabolite with a 5-carbon side chain; 3/1C-RFM, hippuric acid and ring fission metabolites with a one or three carbon side chain

* Corresponding author.
E-mail address: alan.crozier44@gmail.com (A. Crozier).
[1] Present address: Glasgow Polyomics, Wolfson Wohl Cancer Research Centre, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK.

http://dx.doi.org/10.1016/j.freeradbiomed.2016.08.001
0891-5849/

(–)-epicatechin over a 72 h period following intragastric administration to Sprague Dawley rats. Identification and quantification of radiolabeled metabolites made use of HPLC-MS^2, with radio counting (RC) using an on-line radioactivity monitor [16], and was aided by the availability of a wide range of authentic references compounds.

2. Material and methods

2.1. Chemicals

[2-^{14}C](–)-Epicatechin (^{14}C-EC), synthesized according to Sharma et al. [17], provided by Mars Incorporated (McLean, VA, USA), was synthesized by Quotient Bioresearch Ltd (Cardiff, UK). (–)-Epicatechin metabolites were synthesized and characterized as described elsewhere [18–20]), and along with 5-(3,4-dihydroxyphenyl)-γ-valerolactone metabolites, were supplied by the Institute of Pharmaceutical Discovery, LLC (Branford, CT, USA). 5-(3′,4′-Dihydroxyphenyl)-γ-hydroxyvaleric acid was provided by Toronto Research Chemicals (Toronto, Ontario, Canada). HPLC grade ethyl acetate, methanol and ethanol were purchased from Rathburn Chemicals (Walkerburn, Borders, UK). HCl, acetic acid, phosphoric acid and formic acid were obtained from Fisher Scientific Ltd (Loughborough, Leicestershire, U.K.). L-Ascorbic acid, *N, N*-dimethylformamide and all other chemicals were supplied by Sigma Aldrich (Poole, Dorset, UK).

2.2. Animals and husbandry

The study which used 27 male Sprague Dawley rats, age 8–9 weeks, mean body weight 338 g at dosing, was carried out under UK Government Home Office License at the facilities of Charles River Laboratories (Tranent, East Lothian, UK). The animals were acclimatized in the experimental unit for 7 days before supplementation and, during this period, were observed carefully to ensure that they were in good health and suitable for inclusion in the study. Experiments were performed following European Community animal experiment ethical regulations.

During the pretrial holding period, rats were multiply housed in solid bottomed cages with bedding/nesting material and the animals were offered wooden chew sticks for environmental enrichment. During the on-study periods, they were housed individually in metabolic cages specially designed for the separate quantitative collection of urine and feces. The remaining rats were housed in pairs in polypropylene and stainless steel cages with raised wire mesh floors. On arrival of the animals 7 days pre-^{14}C-EC administration until completion of the study, the rats were fed a low polyphenol diet, (AIN-93G Growth Purified Diet) (BCP IPS Ltd, London, UK). Domestic mains tap water was also available ad libitum.

2.3. Feeding protocol

The ^{14}C-EC (specific activity 15.4 μCi/μmol) in 8% aqueous ethanol was administered by gastric gavage to 24 rats, at a volume of 500 μL/rat, to achieve an intake of 20 μCi (44×10^6 dpm, 1.3 μmol, [377 μg]). Three more animals were used as a control. Urine and feces were collected quantitatively from individual animals periodically 0–72 h post-dose. The cages were washed with water at the time of each fecal collection and the washings retained for analysis.

Three rats were killed humanely by CO_2 narcosis at each time point 0–72 h post-administration. Following sacrifice, a terminal blood sample (~5–10 mL) was collected in tubes containing lithium heparin. Blood was centrifuged at 2000*g*, for 10 min and the red blood cells and plasma were retained separately. The whole body tissues were perfused in situ with chilled 0.15 M NaCl to remove residual blood, after which brain, heart, lungs, kidneys, liver, testis, spleen and gastrocnemius muscle were removed, rinsed in saline, blotted dry, and stored at –80 °C. The gastrointestinal tract (GIT) was removed intact together with its contents, then separated into stomach, duodenum, jejunum/ileum, cecum and colon, before being frozen. Feces and urine were collected from each rat during the course of the study and stored at –80 °C. Before analysis, tissues and feces were lyophilized, weighed, and ground to a powder.

2.4. Measurement of total radioactivity in tissues and body fluids

Ten mg aliquots of powdered freeze-dried tissues and voided feces, and 100 μL samples of plasma and urine were treated overnight with 0.5 mL of tissue solubilizer (National Diagnostics, Hull, UK) in a shaking water bath at 50 °C. The resultant clear solutions were mixed with 5 mL scintillation cocktail (National Diagnostics, Hull, UK) and radioactivity determined using a liquid scintillation counter (Beckman Coulter, Fullerton, CA, USA).

2.5. Preparation of tissues and feces for HPLC-RC-MS^2 analysis

The size of aliquots taken for extraction was determined by the amount of radioactivity in the sample with a minimum of 20×10^3 dpm required for analysis by HPLC-RC-MS. Extracts containing a low concentration of radioactivity were not further processed. Feces and tissues were extracted using a method described by Serra et al. [20]. Freeze-dried samples were added to 50 μL of 1% aqueous L-ascorbic acid and 100 μL of 4% aqueous phosphoric acid and first extracted with 800 μL of water/methanol/4% aqueous phosphoric acid (94/4.5/1.5, v/v/v) using a sonicator (Digital Sonifier® model S-150D ultrasonic cell disruptor, Branson, Teltow, Germany) for 30 s and maintained on ice to avoid heat. The extracts were then centrifuged at 16,100*g* for 15 min at 5 °C. The supernatant was decanted and the pellet re-extracted three more times as described above. The four supernatants were combined, diluted with 4 mL of 4% aqueous phosphoric acid and loaded onto an OASIS® HLB cartridge (3 mL, 60 mg) (Phenomonex, Macclesfield, UK) previously conditioned with methanol and 0.2% aqueous acetic acid. The cartridge was washed with 1 mL of 4% aqueous phosphoric acid and 1 mL of 0.2% aqueous acetic acid. The retained compounds were eluted with 2 mL acetone/water/acetic acid (70:29.5:0.5, v/v/v). The eluate was reduced to dryness using a SPS Speedvac concentrator (Thermo Savant, Waltham, MA) and resuspended in 25 μL of methanol containing 0.1% formic acid to which 225 μL of 0.1% aqueous formic acid was added. Extracts were centrifuged at 16,100*g* for 10 min at 4 °C in a 0.2 μm Micro-Spin™ Eppendorf filter (Alltech Associates Applied Sciences, Lancashire, UK) prior to analysis.

2.6. Preparation of plasma for analysis

Plasma was defrosted and 200 μL aliquots was loaded onto a 1 mL Phree Phospholipid Removal cartridge for protein precipitation and phospholipid removal (Phenomenex, Torrance, CA). Dropwise, 800 μL of 1% formic acid in acetonitrile was mixed with the plasma on top of the cartridge which was then filtered under pressure before a second filtration with 400 μL of 1% formic acid in methanol was applied. The eluted samples were concentrated by using a SPS Speedvac concentrator before being resuspended in 25 μL of 0.1% formic acid in methanol and 225 μL of 0.1% aqueous formic acid and centrifuged at 16,100*g* for 10 min at 4 °C prior to analysis by HPLC-RC-MS^2.