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Original Contribution

Structurally related (−)-epicatechin metabolites in humans: Assessment using de novo chemically synthesized authentic standards

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ABSTRACT

Accumulating data suggest that diets rich in flavanols and procyanidins are beneficial for human health. In this context, there has been a great interest in elucidating the systemic levels and metabolic profiles at which these compounds occur in humans. Although recent progress has been made, there still exist considerable differences and various disagreements with regard to the mammalian metabolites of these compounds, which in turn are largely a consequence of the lack of availability of authentic standards that would allow for the directed development and validation of expedient analytical methodologies. In this study, we developed a method for the analysis of structurally related flavanol metabolites using a wide range of authentic standards. Applying this method in the context of a human dietary intervention study using comprehensively characterized and standardized flavanol- and procyanidin-containing cocoa, we were able to identify the structurally related (−)-epicatechin metabolites (SREM) postprandially extant in the systemic circulation of humans. Our results demonstrate that (−)-epicatechin-3′-β-D-glucuronide, (−)-epicatechin-3′-sulfate, and a 3′-*O*-methyl-(−)-epicatechin-5/7-sulfate are the predominant SREM in humans and further confirm the relevance of the stereochemical configuration in the context of flavanol metabolism. In addition, we also identified plausible causes for the previously reported discrepancies regarding flavanol metabolism, consisting, to a significant extent, of interlaboratory differences in sample preparation (enzymatic treatment and sample conditioning for HPLC analysis) and detection systems. Thus, these findings may also aid in the establishment of consensus on this topic.

Flavanols and their oligomeric derivatives, the procyanidins, belong to a subclass of flavonoids widely present in the human diet, particularly in food and beverages such as tea, wine, cocoa, apple, plums, pomegranates, and berries [1–3]. Accumulating data from medical anthropological [4], epidemiological [5–7], and dietary intervention studies [8–15] support the notion that the consumption of a diet rich in flavanols and procyanidins decreases the incidence of morbidity and mortality from cardiovascular diseases. This concept has been further substantiated by recent studies demonstrating that the presence of individual flavanols, such as (−)-epicatechin, in food can, at least in part, be causally linked to the beneficial vascular effects observed after consumption of flavanol- and procyanidin-containing foods [16–18]. In this context, there is an increasing interest in elucidating the mechanisms by which the consumption of these compounds, in particular (−)-epicatechin, mediates the observed effects [19]. However, ingested (−)-epicatechin is metabolized into a wide range of metabolites, including structurally related (−)-epicatechin metabolites (SREM)[1], which maintain an intact flavanol ring, and ring-fission metabolites, originating from (−)-epicatechin breakdown by gut microbiome. Therefore, it is essential to elucidate the specific chemical structures and levels of (−)-epicatechin metabolites that are systemically present in humans, as these metabolic derivatives may represent the molecules actually eliciting the effects observed after (−)-epicatechin consumption [17,18]. In this context, and considering that the vascular effects observed after (−)-epicatechin intake are temporally and quantitatively correlated with SREM in circulation [17,18], establishing the chemical structure of this particular group of metabolites is critical.

There are several studies that have reported on the absorption and metabolism of (−)-epicatechin in humans and other mammalian species [17,20–24]. Thus far, it has been clearly established that ingested (−)-epicatechin is extensively metabolized into SREM by O-methylation, O-sulfonation, O-glucuronidation, and combinations thereof [25–28]. Consequently, the potential postprandial profile of SREM is of considerable broadness with regard to the properties of its individual constituents in terms of chemical structure, molecular

Abbreviations: bw, body weight; SPE, solid-phase extraction; UVD, ultraviolet absorbance detection; FLD, fluorescence detection; ECD, electrochemical detection; MS, mass spectroscopy; aSL, arylsulfatase; βGL, β-glucuronidase; SREM, structurally related (−)-epicatechin metabolites.

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charge, molecular mass, lipophilicity, acidity, chemical reactivity, protein-binding capacity, and others. Thus, it may not be surprising that there exist discrepancies in the literature with regard to the precise structure and abundance of the main SREM systemically present in humans [17,21,23]. Although several factors can be postulated to contribute to these discrepancies (e.g., food matrix effects on absorption, intake amount-dependent differences, interindividual variations), at this point in time, a significant part of the causes underlying this issue may essentially lie in methodological differences, such as sample preparation and chromatographic analyses. Current sample preparation and conditioning methods vary greatly across studies [24,25,29–33], as do the chromatography and detection systems [23,33,34]. Consequently, when considering the broadness of the potential SREM spectrum, the wide-ranging interlaboratory differences in sample preparation and analytical methods may represent a key contributing factor in the substantial differences with regard to the reported levels and relative abundance of particular metabolites. This argument is further supported by considering that none of the currently published methods of either sample preparation or chromatography was validated against an array of authentic standards that reflects the postprandial SREM spectrum in terms of its broad range of physicochemical properties. Consequently, the numerical values for systemic levels of SREM that are currently reported in the literature carry a significant burden of uncertainty. On the surface this issue may seem of a somewhat specialized technical nature, but on the contrary, the correct identification of (−)-epicatechin metabolites and their consistent and accurate measurement across investigator groups is essential not only for characterizing and elucidating the potential metabolites driving (−)-epicatechin-mediated bioactivities in humans, but also for meaningfully assessing dietary intake, investigating cause–effect relationships with regard to potential health benefits, and thus ultimately translating our collective knowledge into recommendations for primary and secondary prevention, dietary guidelines, and public health.

In this study, using a wide range of de novo chemically synthesized authentic (−)-epicatechin metabolite standards, we developed a method to assess the postprandial profile of SREM extant in the systemic circulation of humans. This methodology was subsequently applied in the context of a controlled dietary intervention study in healthy male adult volunteers. The results obtained were evaluated and are discussed in the context of previous data.

Materials and methods

Materials

Authentic, chemically de novo synthesized (−)-epicatechin metabolite standards, including 3′-*O*-methyl-(−)-epicatechin, 4′-*O*-methyl-(−)-epicatechin, and the ammonium salts of (−)-epicatechin-4′-sulfate, (−)-epicatechin-3′-sulfate, (−)-epicatechin-5-sulfate, (−)-epicatechin-7-sulfate, (−)-epicatechin-7-β-D-glucuronide, (−)-epicatechin-3′-β-D-glucuronide, 3′-*O*-methyl-(−)-epicatechin-7-β-D-glucuronide, 4′-*O*-methyl-(−)-epicatechin-5-β-D-glucuronide, 4′-*O*-methyl-(−)-epicatechin-7-β-D-glucuronide, 4′-*O*-methyl-(−)-epicatechin-3′-β-D-glucuronide, and 3′-*O*-ethyl-(−)-epicatechin (recovery standard) were provided by Mars, Inc. (Hackestown, NJ, USA). (−)-Epicatechin and sulfatase and β-glucuronidase enzymes were purchased from Sigma (Saint Louis, MO, USA). Water, *N,N*-dimethyl formamide, methanol, and acetonitrile HPLC grade were purchased from Fisher (Pittsburgh, PA, USA).

Sample preparation for the identification and quantification of individual (−)-epicatechin metabolites in human plasma

The sample preparation method was adapted from Unno et al. [32] and further developed. One milliliter of plasma was spiked with 50 μl of a solution containing 10 μM 3′-*O*-ethyl-(−)-epicatechin (recovery standard) and diluted with 2 ml of 3.4% (w/v) phosphoric acid. Thereafter, samples were loaded onto solid-phase extraction (SPE) cartridges (Oasis HLB 60 mg, 3 cc) previously conditioned with 1 ml of *N,N*-dimethyl formamide (DMF):methanol (7:3) and 0.5% (v/v) acetic acid in water. The washing steps consisted of 3 ml of 0.5% (v/v) acetic acid in water, 1 ml of water:methanol:acetic acid (80:20:0.5), and 1 ml of 0.5% (v/v) acetic acid in acetonitrile. For elution, cartridges were dried and eluted with the addition of 1 ml of DMF:methanol (7:3) twice. The eluate was collected in tubes containing 200 μl of 0.5% (v/v) acetic acid in methanol. The total volume was reduced to approximately 50 μl using a Speedvac concentrator (Thermo Electron Corp., SPD131DDA-115) at 2–3 mm Hg pressure and refrigerated vapor trap (Thermo Electron Corp., RVT4101-115) working at −100 °C. Samples were then mixed with a solution containing 75 pmol of catechol and 300 pmol of resorcinol (internal standards) and analyzed by HPLC within 24 h.

To determine the extraction efficiency (i.e., recovery) of (−)-epicatechin metabolites, plasma samples were spiked with increasing concentrations of the (−)-epicatechin metabolites to generate final concentrations ranging from 10 to 1000 nM. These samples were analyzed applying the method described above. For comparison purposes, plasma samples spiked with select (−)-epicatechin metabolites were analyzed using a different sample conditioning method, which was based on protein precipitation with methanol as described elsewhere [35].

Sample preparation for the quantification of (−)-epicatechin metabolites in human plasma using differential hydrolysis

To assess the presence of those (−)-epicatechin metabolites for which we do not have standards and to further compare the results obtained using the direct quantification of individual metabolites, we quantified (−)-epicatechin metabolites in plasma using differential hydrolysis. This method is based on the treatment of plasma samples with arylsulfatase (aSL) and β-glucuronidase (βGL), enzymes that specifically hydrolyze (−)-epicatechin sulfates and glucuronides, respectively, giving rise to (−)-epicatechin, 3′-*O*-methyl-(−)-epicatechin, and 4′-*O*-methyl-(−)-epicatechin that are later quantified in the samples. Using this approach it is possible to identify the following groups of (−)-epicatechin metabolites: (−)-epicatechin glucuronides, 3′-*O*-methyl-(−)-epicatechin glucuronides, 4′-*O*-methyl-(−)-epicatechin glucuronides, (−)-epicatechin sulfates, 3′-*O*-methyl-(−)-epicatechin sulfates, and 4′-*O*-methyl-(−)-epicatechin sulfates.

To select suitable aSL and βGL enzymes to be used in this study, we compared the extent of hydrolysis of surrogate O-glucuronidated and O-sulfonated metabolites (4-nitrophenyl-β-D-glucuronide and nitrocatechol sulfate, respectively) in plasma using aSL and βGL from: (i) *Helix pomatia* (13.1 kIU/ml of βGL and 0.32 kIU/ml of aSL), (ii) agglutinin-free *H. pomatia* (9.0 kIU/ml of βGL and 0.04 kIU/ml of aSL), (iii) *Escherichia coli* (9.8 kIU/ml of βGL) and abalone entrails (0.14 kIU/ml of aSL), and (iv) *Patella vulgata* (22.1 kIU/ml of βGL and 0.39 kIU/ml of aSL) (Fig. 1). The results obtained demonstrated that aSL and βGL from *H. pomatia* exerted a complete hydrolysis of the surrogate metabolites. In addition, these enzymes were capable of hydrolyzing a series of authentic (−)-epicatechin metabolite standards, including (−)-epicatechin-4′-sulfate, (−)-epicatechin-7-β-D-glucuronide, 3′-*O*-methyl-(−)-epicatechin-7-β-D-glucuronide, 4′-*O*-methyl-(−)-epicatechin-5-β-D-glucuronide, 4′-*O*-methyl-(−)-epicatechin-7-β-D-glucuronide, and 4′-*O*-methyl-(−)-epicatechin-3′-β-D-glucuronide (data not shown). Importantly, although β-glucuronidase from bovine liver was effective at catalyzing the complete hydrolysis of O-glucuronidated metabolites, O-sulfates remained unaffected by these enzymes.

The quantification of (−)-epicatechin, 3′-*O*-methyl-(−)-epicatechin, and 4′-*O*-methyl-(−)-epicatechin in plasma was determined in 0.5 ml