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Fate of Microbial Metabolites of Dietary Polyphenols in Rats: Is the Brain Their Target Destination?

Mattia Gasperotti,[†] Sabina Passamonti,*[,‡] Federica Tramer,[‡] Domenico Masuero,[†] Graziano Guella,[§] Fulvio Mattivi,[†] and Urska Vrhovsek*[,†]

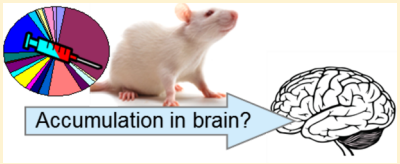
[†]Department of Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach (FEM), via E. Mach 1, 38010 San Michele all'Adige, Italy

[‡]Department of Life Sciences, University of Trieste, via L. Giorgieri 1, 34127 Trieste, Italy

[§]Department of Physics, University of Trento, via Sommarive 14, 38123 Trento, Italy

Supporting Information

ABSTRACT: Different polyphenol compounds are ingested when consuming a serving of fruits rich in polyphenols, spanning from one-phenol hydroxybenzoic acid to more complex polymeric compounds. Only a minor quantity of the polyphenols (5−10%) is absorbed. The remainder reaches the colon and is extensively metabolized by gut microbiota to low-molecular weight metabolites. Their subsequent tissue distribution is still undefined, although these microbial metabolites are currently believed to play a role in human health and disease states. To fill this knowledge gap, we performed a pharmacokinetics experiment in which a single bolus of 23 polyphenol microbial metabolites (total 2.7 μmol) was administered intravenously to rats to reliably reproduce a physiological postabsorption situation. Tissues and urine were collected shortly thereafter (15 s to 15 min) and were analyzed by UHPLC-MS/MS to quantitatively track these compounds. Remarkably, the brain was found to be a specific target organ for 10 of the 23 polyphenol metabolites injected, which significantly increased in the treated animals. In most cases, their appearance in the brain was biphasic, with an early wave at 2 min (4 compounds) and a second wave starting at 5 min; at 15 min, 9 compounds were still detectable. Most compounds were excreted into the urine. The concentrations in the brain of the treated animals were compared against those of the control group by Student's *t* test, with *p*-values < 0.1 considered to be statistically significant. These findings provide new perspectives for understanding the role of diet on brain chemistry. Our experimental approach has enabled us to obtain rich metabolomics information from a single experiment involving a limited number of animals.



KEYWORDS: *Polyphenols, gut microbiota, target metabolomics, mass spectrometry, brain, fruit consumption*

A diet rich in fruit and vegetables is regarded to be an important factor for protecting the human organism from chronic diseases.[1] However, dietary recommendations require knowledge about food composition, identification of bioactive food components, and characterization of bioavailability and tissue distribution, the elimination patterns of metabolites, and components' specific effects on cellular homeostasis.

Polyphenols, which are a wide and heterogeneous group of bioactive compounds found in vegetables and fruits,[2] especially berries,[3] are regarded as being responsible for protecting the human organism from oxidative stress-related chronic diseases. The bioactivity of polyphenols may serve a much broader purpose, however.

After ingestion, only a minor part of the polyphenols (5−10%) is absorbed in the small intestine. The remainder reaches the colon, where it is extensively metabolized by gut microbiota to low-molecular weight compounds.[4] Gut microbiota consist of many trillions of microbial cells[5] whose metabolic activities have been shown to modulate human metabolic phenotypes[6−8] and thus play an essential role in human health.

The microbiota break down dietary polyphenols through the actions of glucosidases, esterases, demethylases, dehydroxylases, and decarboxylases[5,9−18] to produce smaller common metabolites, such as phenolic acids and short-chain fatty acids,[9] collectively known as polyphenol microbial metabolites (PMMs). These are absorbed by the colonic epithelium and are found in the blood with patterns that correlate with the diet and individual microbial composition.[19]

PMMs are regarded as being responsible for the health effects that correlate with regular berry fruit consumption.[4,20] Epidemiological studies associate polyphenol consumption with reduced cancer and cardiovascular disease risk.[21,22] Research on the neuroprotective effects and prevention of brain aging by dietary polyphenols has also increased in recent years.[22−28] The literature reports that diets rich in polyphenols attenuate neuropathology indicators and cognitive decline.[25] Some studies report direct effects of polyphenols on intracellular targets, e.g., three members of the secretase family known to be involved in the amyloidal aggregation have been connected with the onset and progression of Alzheimer's disease.[29] The question

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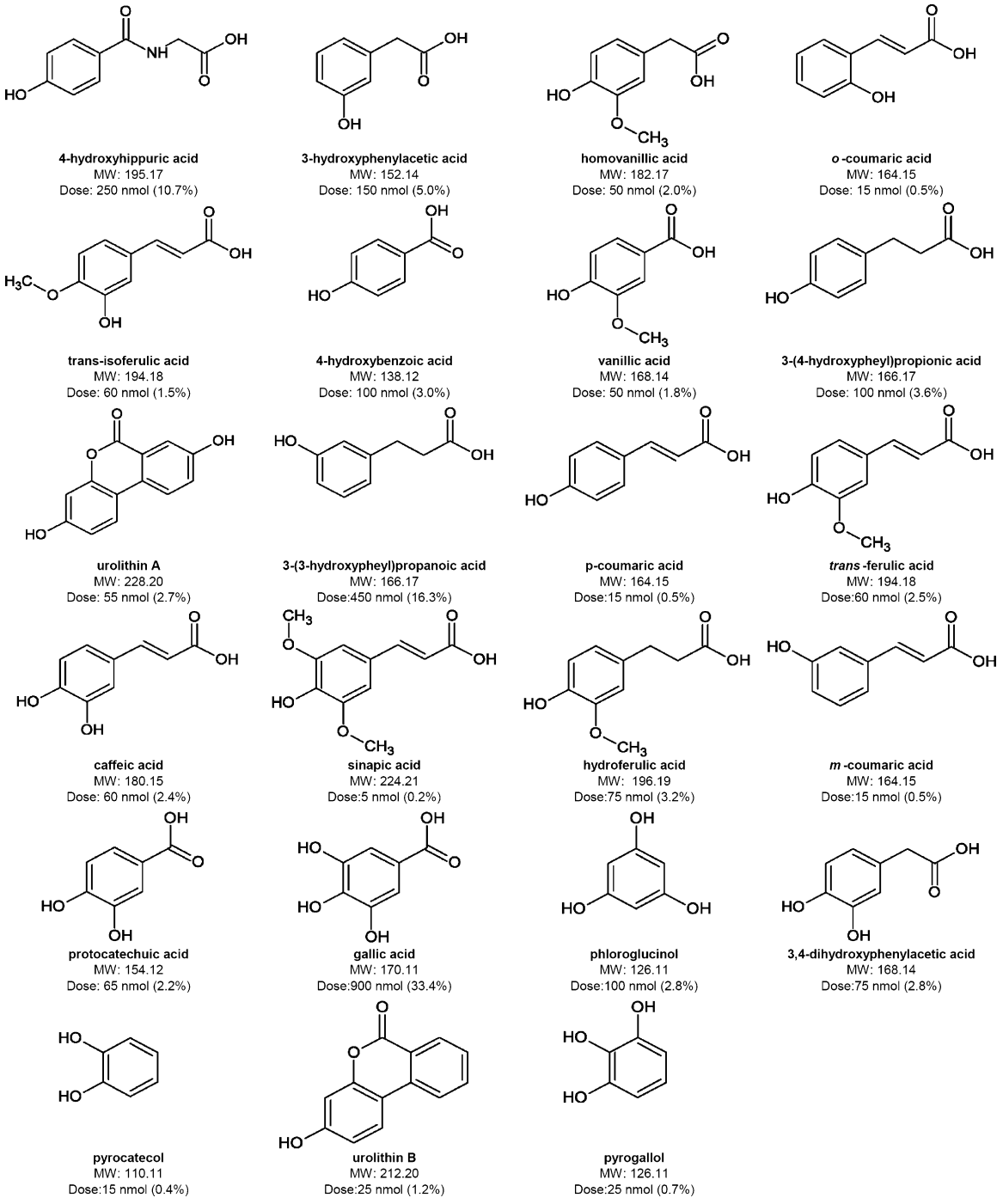

Figure 1. Mixture of polyphenol microbial metabolites used for the intravenous injection, their chemical formula, molecular weight (MW), dose, and relative percentage of the total amount injected, 2690 nmol (dosage: 168.13 μM).

of whether these compounds are able to pass the blood–brain barrier and achieve a pharmacologically relevant concentration there remains unanswered.

The importance of microbiota for brain function development has been noted by numerous studies of experimental animals,[30−32] and these observations can be translated to a better understanding of human brain disorders. For instance, autism, a defect of brain development leading to impaired social interaction, is now understood to be linked to abnormal microbiota population and poor intake of fruits and vegetables.[33] It might be speculated that PMMs are involved in normal neural development and function. Indeed, they are chemically identical or similar to catabolites of amino acids and neurotransmitters.

It seems, however, that PMMs may have some yet uncharacterized bioactivity that has an impact on health.[20] Current information concerning the absorption and distribution of PMMs in mammalian tissues is still poor[9,34] and not substantial enough to support the hypothesis that PMMs are the bioactive agents of our diet. Therefore, it is not possible to make dietary recommendations for early prevention of brain dysfunctions.

Given this background, the focus of this study was to examine the ability of selected, unconjugated PMMs to enter the brain

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Table 1. Percentage of Recovery in Blood Compared to the Initial Injected Dose and Pharmocokinetic Analysis of Polyphenol Microbial Metabolites[a]

	time points (min)												pharmacokinetics		
	dose	t_0[b]	$t_{0.25}$		t_2		t_5		t_{15}		control		steady state ($t = \infty$)	$AUC_{(0\rightarrow15)}$	k_e
	nmol	%	average recovery % ± SD										pmol/mL	pmol[b] min/mL	min^{-1}
4-hydroxyhippuric acid	250	**13.3**	11.7	5.5	13.4	6.2	6.4	6.1	2.4	0.6	0.0	0.0	**0.00**	**15 017**	**0.09**
3-hydroxyphenylacetic acid	150	**19.9**	18.6	6.2	10.5	2.7	6.5	1.5	3.0	1.6	0.6	0.2	**304.50**	**9238**	**0.38**
homovanillic acid	50	**18.7**	17.5	5.2	8.8	0.8	7.0	1.7	3.0	1.2	0.4	0.3	**118.40**	**3022**	**0.45**
o-coumaric acid	15	**34.6**	32.0	11.1	15.7	2.3	12.1	2.8	5.2	2.3	0.1	0.1	**62.70**	**1597**	**0.47**
trans-isoferulic acid	35	**25.9**	23.2	7.1	9.3	1.8	7.0	2.3	1.9	1.0	0.0	0.0	**73.40**	**2125**	**0.58**
4-hydroxybenzoic acid	100	**18.5**	16.3	5.3	6.5	0.6	3.8	1.5	1.5	0.4	1.7	0.9	**131.50**	**3864**	**0.62**
vanillic acid	50	**18.7**	16.3	7.6	6.4	1.8	3.9	1.5	1.8	0.4	1.3	0.5	**75.70**	**1992**	**0.67**
3-(4-hydroxyphenyl) propanoic acid	100	**24.8**	21.9	6.4	10.1	1.0	8.4	2.2	4.6	1.1	0.4	0.5	**371.50**	**7538**	**0.69**
urolithin A	55	**11.5**	9.7	3.3	2.9	0.9	0.8	0.1	0.1	0.1	0.0	0.0	**9.80**	**733**	**0.71**
3-(3-hydroxyphenyl) propanoic acid	450	**15.5**	13.5	3.6	5.7	0.8	5.1	0.8	2.2	0.6	0.8	0.6	**929.30**	**19 569**	**0.72**
p-coumaric acid	15	**22.2**	19.3	5.0	7.9	1.3	6.6	1.3	3.0	1.1	3.3	5.4	**40.50**	**872**	**0.74**
trans-ferulic acid	60	**18.0**	14.7	5.9	3.8	0.5	1.9	0.7	0.5	0.5	0.0	0.1	**39.30**	**1387**	**0.88**
caffeic acid	60	**1.8**	1.4	0.5	0.3	0.0	0.1	0.1	0.0	0.1	0.0	0.0	**2.53**	**108**	**0.95**
sinapic acid	5	**14.5**	11.6	4.8	2.8	0.3	1.2	0.1	0.6	0.7	0.0	0.0	**2.40**	**84**	**0.95**
hydroferulic acid	75	**19.3**	15.7	5.7	4.6	0.4	3.6	1.2	1.2	0.7	0.1	0.1	**105.30**	**2535**	**0.96**
m-coumaric acid	15	**43.5**	35.1	12.6	9.8	1.5	6.8	2.9	2.7	1.8	0.0	0.0	**41.60**	**1044**	**0.97**
protocatechuic acid	65	**8.5**	6.6	1.2	3.3	0.5	4.0	3.9	2.4	0.7	1.4	0.1	**129.70**	**2101**	**1.72**
gallic acid	900	**1.0**	0.6	0.5	0.1	0.0	0.1	0.1	0.1	0.0	0.0	0.0	**72.40**	**1296**	**2.73**
phloroglucinol	100	**26.3**	23.0	5.8	26.2	4.1	22.8	10.8	17.7	9.8	17.0	6.7	[c]	**19951**	[c]
3,4-dihydroxyphenyl acetic acid	75	[c]	0.3	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	[c]	**13**	[c]
pyrocatechol	15	[c]	1.0	0.9	1.2	2.4	0.0	0.0	0.0	0.0	0.0	0.0	[c]	**35**	[c]
urolithin B	25	[c]	1.3	1.0	0.4	0.2	9.4	4.2	9.3	12.4	0.0	0.0	[c]	**1721**	[c]

[a]Dose at the steady state ($t = \infty$), area under the curve ($AUC_{0\rightarrow15}$), and elimination rate (k_e) are given. For the recovery, the values given are the percentages of dose found in rats after the i.v injection at four different time points ($t_{0.25}$, t_2, t_5, t_{15min}) and control; SD is the standard deviation of four replicates. For t_0, the percentage reported is an extrapolation based upon the pharmacokinetics curves associated with a first-order exponential decay for an i.v. injection. [b]Extrapolated value from the pharmacokinetic curves for the dose at time zero. [c]No available extrapolated data due to nonfitting with the proper pharmacokinetic curves associated with an i.v. injection.

relative to the main excretory organs. Therefore, we performed an experiment to assess the time-dependent tissue distribution of a single intravenous injection of a PMM mixture in anaesthetized rats.

The superior selectivity, sensitivity, and dynamic range of modern tandem mass-spectrometry enabled us to harvest data on a wide range of diverse PMMs in a single *in vivo* experiment. It is noteworthy that our experimental strategy involved the least number of animals, in compliance with the 3R principles on animal experimentation.

The data obtained show, for the first time, the simultaneous quantitative profile of several PMMs in a mammalian system, proving their distribution in the brain, the main excretory organs (liver and kidneys), the heart, the blood, and the urine. For some PMMs, specific tropisms were also observed, pinpointing target organs for future experimentation. Remarkably, the data obtained show that at least 10 PMMs incrementally appear in the brain within 15 min, whereas they simultaneously disappear from the blood and/or reach other organs.

RESULTS AND DISCUSSION

The Tested Mixture of Polyphenol Microbial Metabolites: A Rational Choice. The strategy was to simultaneously inject 23 metabolites in a well-defined mixture into a single animal (Figure 1).

The selected metabolites are known products of the gut microbiota metabolism of polyphenols found in berries.[11−13,17,35] Their individual concentrations in the mixture were chosen to mimic their average plasma concentration measured after the intake of a berry fruit serving.[36] All individual molecules were commercially available except for urolithin A and B. The latter are regarded as specific biomarkers of dietary ellagitannins metabolized by gut microbiota and were synthesized in-house.[37]

An analytical method based on UHPLC-MS/MS enabled quantitative measurements of this set of 23 compounds in biological fluids and tissue extracts from a single rat,[38] resulting in a remarkable reduction in the number of experimental animals, as recommended by the regulatory bodies.[39]

The *in vivo* injection of a multicomponent mixture could give rise to potential problems. Some metabolic pathways could transform certain metabolites into other compounds already present in the mixture, thus altering the chemical composition of the injected mixture. The simultaneous injection of different metabolites could also affect the rate of membrane transport, enzyme pathways, or cellular regulatory mechanisms, resulting in competitive interactions, rapid states of saturation, or coopera-

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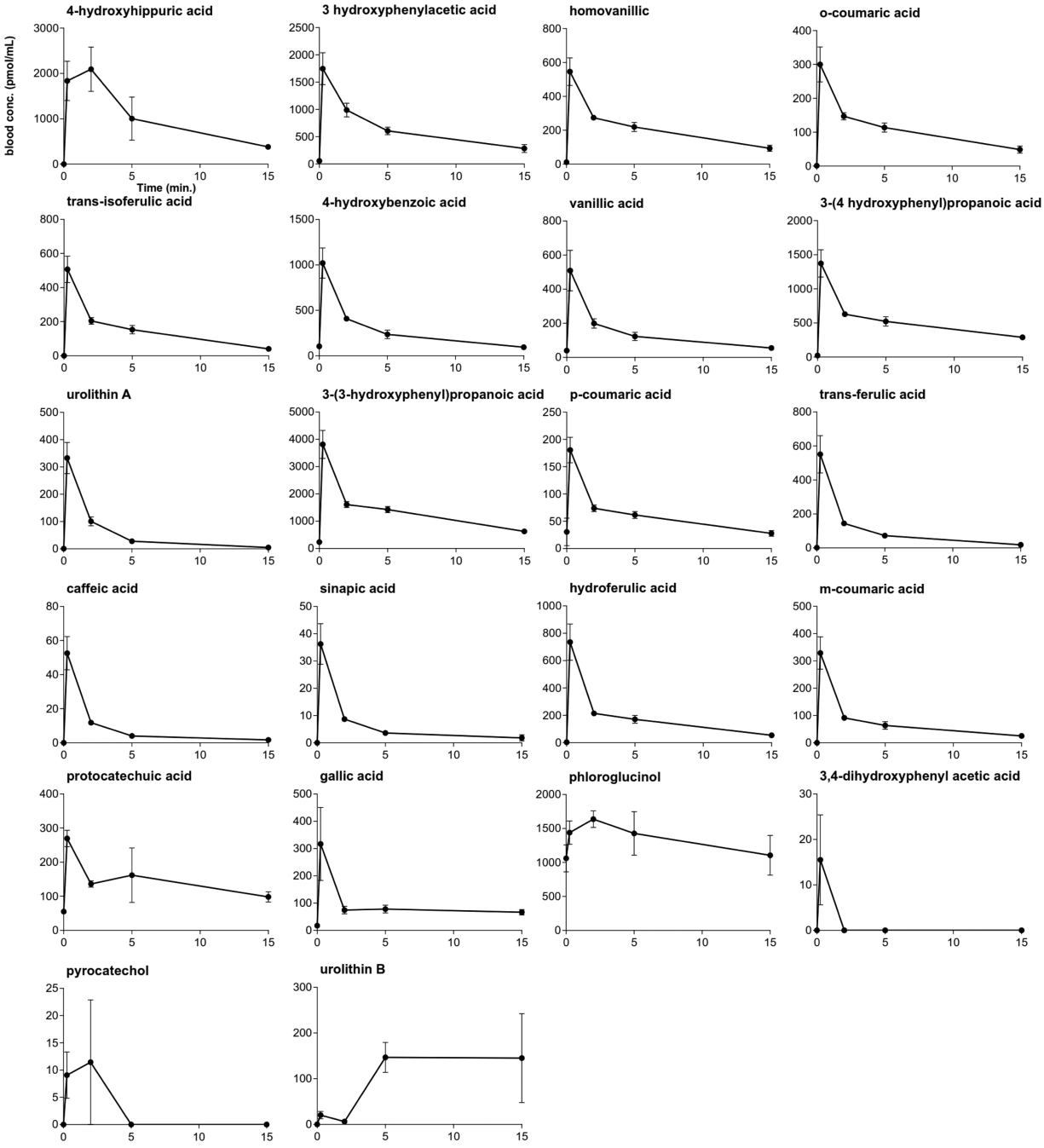

Figure 2. Kinetics of distribution of polyphenol microbial metabolites in blood.

tive responses, to name a few. However, this was a physiological situation, closely simulating a nutritionally relevant scenario in the blood. Analyzing a multicomponent mixture made it possible to simultaneously follow the tissue distribution of each compound, especially with respect to the brain, whereas the presence of other congeners or further metabolites (e.g., glucuronides or sulfates) could not be followed.

Dosage, Administration Route, and Time Line: A Strategy for Accurate Tissue Distribution Analysis. The most accurate approach for a study on PMM tissue distribution is to administer the PMMs by intravenous injection, thereby minimizing the main factors of interindividual variability in gut metabolism, motility and absorption.[6] Any other downstream processes, such as distribution into tissues and mammalian phase-1 and phase-2 metabolism, are comparatively more homogeneous among individuals.[40] As a consequence, a satisfactory variation coefficient was obtained for all measured parameters, using the least number of animals (n = 4 in this study).

The injected dose was 2.7 μmol, which was the sum of 23 individual PMM amounts (Figure 1) from 0.9 μmol (gallic acid) to 0.0005 μmol (sinapic acid). Considering that the rats (body mass = 288 ± 20 g) had an estimated blood volume of 16 mL[41] and assuming that all of the mixture components had free access to the internal volume of blood cells, the initial blood concentration was 168 μM, which spanned from 56.25 μM (gallic acid) to 0.31 μM (sinapic acid). These values are within the concentration range observed in humans following the consumption of a standard serving of berry fruits.[42]

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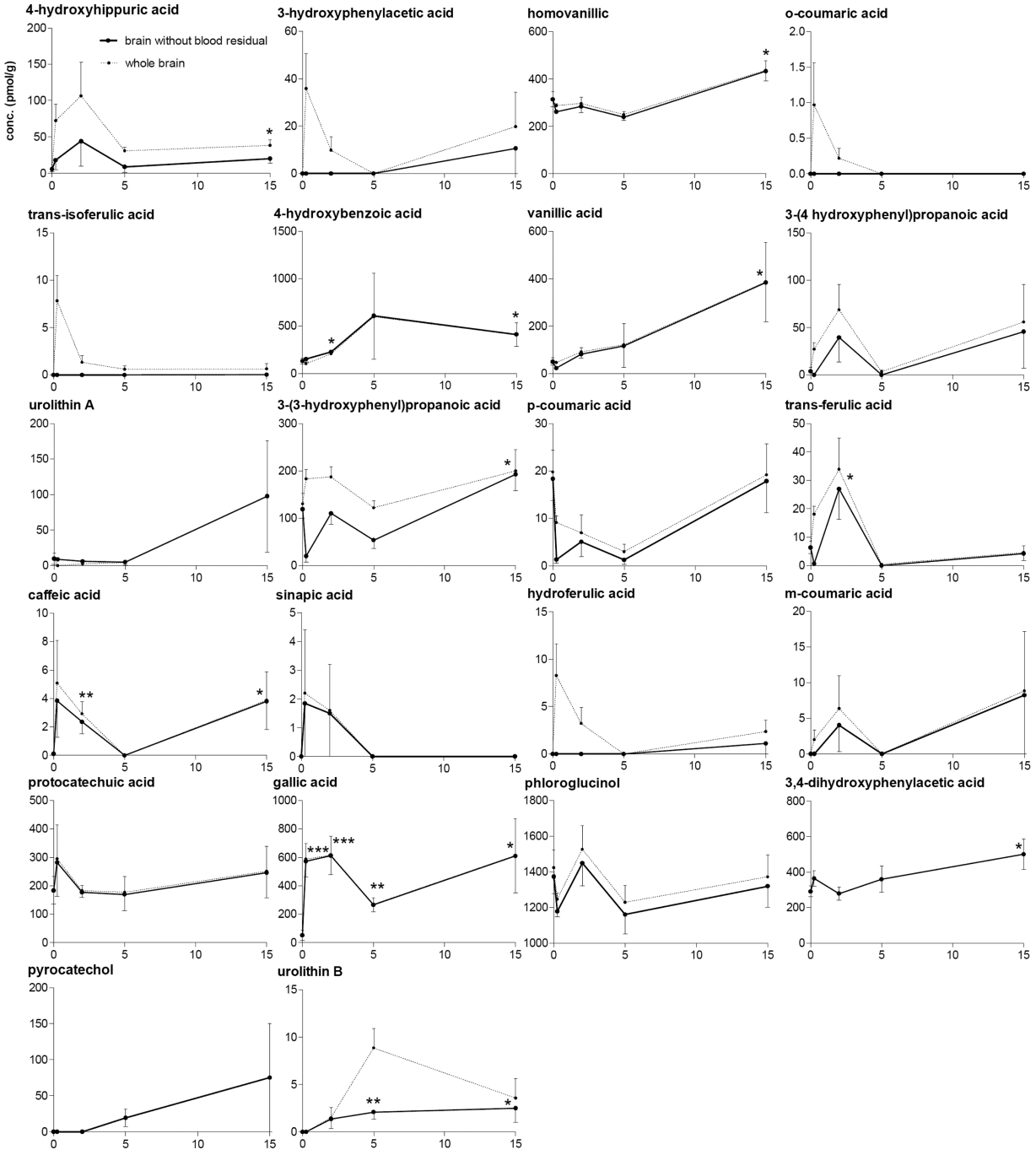

Figure 3. Kinetics of distribution of polyphenol microbial metabolites in brain. Dotted lines are referred to the amount found in the whole brain, whereas continuous lines are the data after subtracting the residual blood in the brain. Data with asterisks are significantly increased from the control samples ($p < 0.01$, ***; $p < 0.05$, **; $p < 0.1$, *).

The experiment was designed on the basis of previous data showing the exceptionally fast uptake and metabolism of a dietary flavonoid, i.e., cyanidin 3-glucoside, a ubiquitous pigment in red fruits.[40] It was assumed that the plasma disappearance kinetics of low-molecular weight flavonoid metabolites and other dietary polyphenols should follow a similar pattern.

The site for the intravenous PMM mix injection was the dorsal penis vein, which is easily accessible through a thin epidermal layer and is afferent to the general circulation.[43] Minimally manipulating an anaesthetized animal is seen as a crucial factor because any inflammation mediator that is locally released following surgical trauma might affect the permeability of the blood−brain barrier and/or the basal functions that determine distribution and excretion (i.e., membrane transporters) or metabolism (i.e., enzymes).

The time points chosen seemed to be appropriate for the time course, both in terms of the injected molecules' disappearance from the blood and their appearance in the organs and urine. Under the chosen conditions, the animals were under minimal duration of anesthesia and physical stress. Overall, this experiment is a refinement of previous protocols that we have used.[40,44]

Presence of Basal Levels of the Mixture Components in Control Animals. Many of the mixture components arise not

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Table 2. Endogenous Amounts of Polyphenol Metabolites in Control Brain as Well as Polyphenol Metabolites That Were Significantly Increased Compared to Those in Control Brain at All Time Points ($p > 0.1$)

	dose[a]	control	$t_{0.25}$	t_2	t_5	t_{15}	fold change t_2/control	fold change t_{15}/control
	pmol/g of animal			pmol/g				
4-hydroxyhippuric acid	868	5.52				20.06		3.63
3-hydroxyphenylacetic acid	521	n.d.						
homovanillic acid	174	314.81				433.16		1.38
o-coumaric acid	52	n.d.						
trans-isoferulic acid	121	n.d.						
4-hydroxybenzoic acid	347	126.63		206.97		407.92	1.63	3.22
vanillic acid	174	99.71				384.71		3.86
3-(4-hydroxyphenyl)propanoic acid	347	3.88						
urolithin A	191	9.72						
3-(3-hydroxyphenyl)propanoic acid	1562	119.01				192.81		1.62
p-coumaric acid	52	18.34						
trans-ferulic acid	208	6.34		27.01			4.26	
caffeic acid	208	0.11		2.36		3.81	21.45	34.64
sinapic acid	17	n.d.						
hydroferulic acid	260	n.d.						
m-coumaric acid	52	n.d.						
protocatechuic acid	226	183.40						
gallic acid	3125	52.17	573.00	612.72	265.97	610.82	11.74	11.71
phloroglucinol	347	1373.36						
3,4-dihydroxyphenyl acetic acid	260	291.82				501.19		1.72
pyrocatechol	52	n.d.						
urolithin B	87	n.d.			2.09	2.50		>2.50
compounds increased at any time ($p > 0.1$)			1	4	2	9		

[a]The dose is expressed as pmol/g of animal considering the rat average weight (288 g) for comparison with the amounts accumulated in rats.

only from the microbial metabolism of dietary polyphenols but also from mammalian catabolism of endogenous substrates.[36] Thus, some were expected to occur in the rat organs and fluids at basal levels in control animals. Compounds that are exclusively products of microbial metabolism are the urolithins, ferulic acid, gallic acid, coumaric acid, and protocatechuic acid.[36] However, these might also be expected to occur in control animals, as they are markers of normal nutritional status (e.g., *trans*-ferulic acid). The basal levels of the 23 component mixture were very low in general. Exceptions are mentioned case-by-case below.

Tracing Polyphenol Metabolites in Blood. The blood of control rats contained 11 of the 23 injected molecules. Of these endogenous compounds (Table 1), six were found at less than 1% of the amount occurring in the injected mixture, and another three were found at less than 2%. The two outliers were *p*-coumaric acid (3.3%) and phloroglucinol (17.0%). Thus, the selected mixture represented a valid challenge for a pharmacokinetic test because of the low basal levels. This test seemed to reliably simulate what happens in the organism after consumption of a standard berry fruit serving after a short wash out period.

Most injected compounds disappeared following apparent pseudo-first-order kinetics, as shown in Figure 2. The calculated parameters, i.e., the extrapolated amounts at time zero (t_0) and at steady state (t_∞), and the disappearance rate constant (k_e), which spanned from 2.73 to 0.38 min^{-1} ($t_{1/2}$ = 0.25 to 1.84 min) and pointed to a rapid process, are shown in Table 1. The recovery in blood extrapolated at t_0 was within 8.5 and 43.5% for 17 of the 23 metabolites (Table 1), showing that a major fraction of these compounds was not easily extractable from the whole blood. The formation of complexes with the serum proteins, therefore, still left a significant concentration of the unbound form; for the majority of the studied metabolites, this is expected to be the active form, which undergoes metabolism and reaches the tissues. However, there were a few exceptions.

Pyrogallol could never be detected, although its recovery and analysis posed no specific issues; the mechanisms of its disappearance were unknown. Two compounds, pyrocathecol and 3,4-dihydroxyphenyl acetic acid, were detected only at 15 s. Urolithin B was detected at very low levels at both 15 s and 2 min and rose thereafter by an order of magnitude. Phloroglucinol occurred at a similar level during the entire experiment. Gallic and caffeic acid showed a very modest recovery, indicating a very modest presence in the free form in the bloodstream.

With the few exceptions mentioned above, steady-state values were attained even earlier than 5 min; therefore, the duration of the experiment was appropriate. In most cases, the compounds had essentially disappeared by 15 min.

The calculated amounts at t_0 and at any time point were always lower than the sum of the injected dose and any endogenous compounds in the control samples. The calculated t_0 amounts ranged from 43.5% (*m*-coumaric acid) to 1% (gallic acid) and were expressed as a percentage of the injected amounts, as shown in Table 1. This result is not surprising given the fast uptake and excretion of one precursor of these metabolites, i.e., the fruit pigment cyanidin 3-glucoside.[40]

An explanation for the loss of part of the injected dose and for the extremely rapid blood disappearance may be quick uptake and distribution to the organs. Only some major organs were collected, so a cumulative recovery assessment could not be provided. The compounds also might have been distributed in large tissues, such as adipose, vascular endothelium, or

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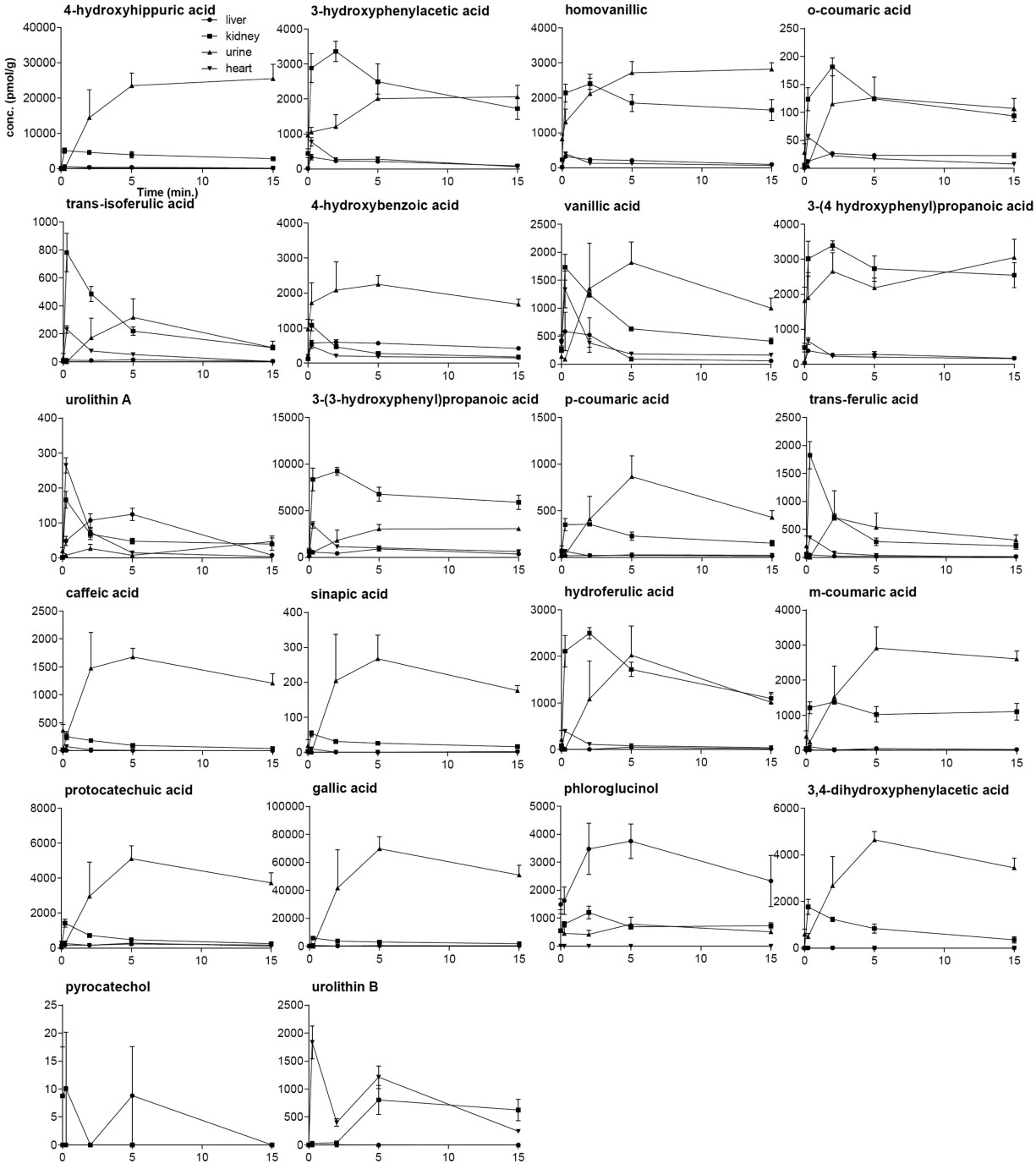

Figure 4. Kinetics of distribution of polyphenol microbial metabolites in liver, kidney, urine, and heart. The legend for the symbols is reported in the first graph (top left).

connective tissues. The administered compounds could also have been metabolically transformed, but these were not analyzed in this experiment. This is the first attempt at a simultaneous kinetic characterization of 23 polyphenol metabolites.

Polyphenol Microbial Metabolites in the Brain. The main findings concern the detection of PMMs in the brain at times when their blood concentrations were vanishingly low (Figure 3). Each plot shows two curves, which represent the total amounts measured in whole brain (dotted line) and those corrected for residual blood (continuous line), as recommended.[45−48] Indeed, the brains were not perfused to wash out the blood because this procedure would have altered the chemical equilibrium between the vascular compartment and the whole brain.

Thirteen of the 23 compounds in the mixture were found in the control brains as endogenous metabolites (Table 2). Disregarding the observation time, 10 of the 23 compounds were found in the brain at a significantly increased amount with respect to that in the controls (Table 2). In most cases, their appearance in the brain was biphasic, with an early wave at 2 min, as observed with four compounds, and a second wave starting at 5 min. Nine compounds were detected at 15 min. Gallic acid, which was the most abundant compound in the mixture (Figure 2), best showed this biphasic accumulation trend. It is

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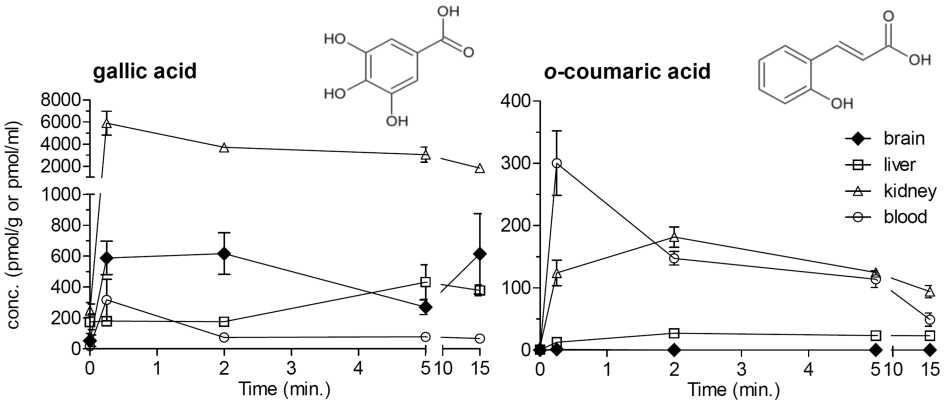

Figure 5. Kinetics of distribution of gallic acid and *o*-coumaric acid in brain, liver, kidneys, and blood.

noteworthy that the gallic acid accumulated in the brain at approximately 11 times the basal concentration measured in the controls as early as 15 s. Similarly, caffeic acid had two peaks at 2 and 15 min and accumulated by approximately 20 and 34 times, respectively (Table 2).

Importantly, two compounds (*trans*-isoferulic acid and *o*-coumaric acid) were neither detected in the brain as endogenous metabolites nor did they ever appear in the brain despite their relatively low plasma elimination rate constant. This proved that the blood−brain barrier was intact during the experiment. Endogenous compounds protocatechuic acid and phloroglucinol never demonstrated an increased concentration in the brain.

The compounds that accumulated in the brain should be active because, in all cases except urolithin B, they were endogenous metabolites already present in the control brains. Their most predictable effect is unbalancing a pre-existing metabolic equilibrium. *trans*-ferulic acid, which accumulated by 15 s in the experiment, is reportedly able to reduce oxidative damage and amyloid pathology in Alzheimer's disease.[49] Some PMMs are identical to neurotransmitter metabolites. For instance, vanillic acid and homovanillic acid are catecholamine catabolites that are found in the brain and in cerebrospinal fluid.[50] Moreover, 3,4-dihydroxyphenyl acetic acid (DOPAC) is a neuronal metabolite of dopamine (via monoamine oxidase) and a direct precursor of homovanillic acid. DOPAC is involved in dopamine catabolism in the pathogenesis of Parkinson's disease.[51,52] Another mechanism of action involves the enzyme kinetics. Caffeic acid, for example, may bind to tyrosine ammonia lyase, thus altering serotonin homeostasis,[53] or it may inhibit acetylcholinesterase and butyrylcholinesterase activities, thereby preventing oxidative stress-induced neurodegeneration.[54] Gallic acid has also been shown to act on key enzyme processes in the brain[26,55] and can accumulate in the brain after repeated doses.[56]

Polyphenol Microbial Metabolites in Other Organs. The analyses of PMMs in the heart, liver, kidneys, and urine are shown in Figures 4 and 5 (and in Supporting Information Figures S1−S4). Eighteen of the 22 compounds attained very high concentrations in the urine (Figure 4 and Supporting Information Figure S1). The exceptions were pyrocatechol and urolithin B, which were not found in the urine; urolithin A, the concentration of which slightly increased; and phloroglucinol, which did not change with respect to the value found in control animals. Thus, most PMMs can be defined as nephrotropic. Indeed, 20 of the 22 compounds dramatically increased their parenchymal concentrations from basal values of approximately zero up to at least 2 orders of magnitude higher (Figure 4 and Supporting Information Figure S2). Due to this extraordinary speed, the apparent rate of accumulation in the kidneys could not be calculated. Accumulation lasted no more than 2 min and often less, after which time the concentration started to decay. In contrast, the appearance in the urine was slower, with a lag phase lasting 15 s before the onset of urinary excretion in most cases (20 out of 22).

Only a few compounds were found to be hepato- rather than nephrotropic, including phloroglucinol, urolithin A, and 4-hydroxybenzoic acid (Figure 4 and Supporting Information Figure S3). One compound (sinapic acid) could not be detected in the liver for three consecutive sampling times even though it was present in the blood. Therefore, the amount of blood retained in the organ was negligible. Similarly, the basal levels of 3-(3-hydroxyphenyl) propanoic acid did not change in the liver, despite its presence in the blood at a 2 orders of magnitude higher concentration.

Only three compounds were found to be cardiotropic, i.e., vanillic acid and both urolithins (Figure 4 and Supporting Information Figure S4). They accumulated to a maximum concentration at 15 s, after which vanillic acid and urolithin A disappeared, whereas urolithin B started to increase again at 5 min. Three compounds (phloroglucinol, 3,4-dihydroxyphenyl acetic acid, and pyrocatechol), although present in the blood, could not be detected in the heart, demonstrating negligible blood contamination during the tissue extraction.

The low apparent hepatotropism of most PMMs leads to the conclusion that PMMs absorbed from the colon under normal conditions undergo limited first-pass metabolism into the liver so that they can be distributed to the other organs readily after absorption. They are then eliminated in the urine after prior accumulation in the kidneys. Only one compound, 4-hydroxybenzoic acid, appeared in the urine at 15 s, likely by glomerular filtration.

The Case of Urolithins. Urolithins, with molecular weights ranging from 212 to 228 Da, are the largest PMM molecules (Figure 1) and arise from the microbial metabolism of ellagitannins and ellagic acid conjugates.[57] Urolithin A differs from urolithin B by one additional hydroxyl group. No other mammalian enzyme pathway is known to produce such end products from more complex precursors. No urolithins were detected in the organs of the control animals. Urolithin A and B were administered at different doses, i.e., 55 and 25 nmol, respectively. Nevertheless, their concentrations in the blood differed by an order of magnitude at 15 s (332.6 and 20.3 pmol/mL, respectively). Thus, urolithin B was sequestered (and/or metabolized) more rapidly than urolithin A in the tissues.

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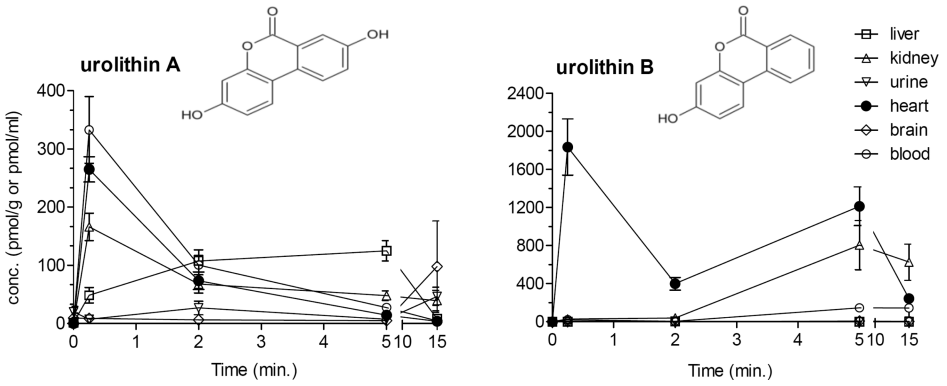

Figure 6. Distribution kinetics of urolithin A and B in all of the organs considered for the experiment.

Both urolithins displayed a unique blood concentration pattern, tissue distribution, and urinary excretion (Figure 6). Indeed, although urolithin A had a normal monoexponential disappearance from the blood, urolithin B increased in the blood after 2 min from a very low level (see above). Therefore, it seems that urolithin A underwent a very unusual and mechanistically unexplained dehydroxylation reaction. Only bacterial dehydroxylases are known.

Both urolithins were negligibly excreted in the urine, which is unique among the PMMs. They were presumably excreted as glucuronyl derivatives,[11] which were not analyzed. Rapid uptake of urolithin A into the kidneys was observed, whereas urolithin B appeared in the kidneys only after 2 min, when it was also present in the blood. The liver slowly absorbed urolithin A up to 5 min, but no urolithin B could be detected in the liver at 15 min. This shows that the liver could neither convert urolithin A to urolithin B nor take the latter up from the blood.

The organ that displayed the highest uptake capacity was the heart, where both urolithin A and B were found at 15 s, at approximately 300 and 2000 pmol/g, respectively. It seems likely that the following occurred: (i) urolithin A was rapidly (by 15 s) taken up into the heart and dehydroxylated to urolithin B by an unknown enzyme(s) or in the heart both urolithins could be converted to other compounds that were not followed; (ii) urolithin B was released from the heart into the blood, where it increased from 2 to 5 min; and (iii) from 5 min on, the urolithin B tissue concentrations increased to higher levels than those in the blood.

Urolithins have a demonstrated activity of reducing protein glycation[58] in neuronal cells and possibly even in the heart. In fact, the advanced glycation end products, derived from the glycation reaction in the heart and vascular tissue, are responsible for a reduction in artery and heart elasticity and have a role in the progression of cardiovascular complications associated with diabetes.[59]

Is the Brain a Target of Polyphenol Metabolites? The findings would suggest so. To our knowledge, ours is the first report showing that no less than 13 PMMs were found at basal levels in control brains. As a consequence, the possibility of their passage through the blood–brain barrier must be assumed *a priori* to ensure exchange with the blood compartment.

Ten of the 23 compounds were found to be significantly increased after injecting the PMM mixture into the animals. Some of the compounds could have a strictly dietary origin, such as gallic acid or *trans*-ferulic acid, whereas others could be catabolites of endogenous compounds, such as catecholamine, dopamine, amino acids, and others. Figure 5 highlights the gallic acid concentration attained in all of the organs considered at various times. The brain clearly had the capacity to extract gallic acid from the blood, which was even higher than that of the liver. On the other hand, the tightness of the blood–brain barrier is demonstrated by its absolute impermeability to *o*-coumaric acid, in spite of its higher hydrophobic index (logP = 2.45, whereas gallic acid's logP is 0.91). However, both gallic acid and *o*-coumaric acid have similar concentrations in the blood.

These data suggest that there are further health implications for the brain–gut microbiota axis[54] and highlight how PMMs could be players in the putative connections/messengers between the microbiota and brain. These bidirectional interactions have already been explored and highlight the importance of the gut microbiota for brain development and behavior,[55] as well as for their influence on anxiety and depression.[56] Because PMMs are the product of dietary polyphenol intake and metabolism by the gut microbiota, it is reasonable to assume that these compounds might be able to regulate human health and disease states.[57] Moreover, the role of gut microbiota in the alteration of mammalian blood metabolite levels has already been demonstrated through combining data from germ-free and conventional animals.[58] Their action may be related to oxidative stress control or, more likely, to the modulation of biochemical and physiological processes by changing the level of endogenous compounds in the brain and other organs, as observed in the present study.

METHODS

Chemicals. Phloroglucinol, pyrogallol, gallic acid, protocatechuic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxyhippuric acid, 4-hydroxybenzoic acid, pyrocatechol, caffeic acid, vanillic acid, 3-hydroxyphenylacetic acid, homovanillic acid, 3-(4-hydroxyphenyl) propanoic acid, 3-(3-hydroxyphenyl)propanoic acid, hydroferulic acid, *trans*-ferulic acid, *trans*-isoferulic acid, sinapic acid, *m*-coumaric acid, *o*-coumaric acid, and *p*-coumaric were purchased from Sigma-Aldrich (Saint Luis, Milan, Italy). The isotopically labeled compounds, butyric acid-d_7 and *trans*-cinnamic acid-d_5, were used as internal standards and purchased from C/D/N Isotopes Inc. (Quebec, Canada). Urolithin A and urolithin B were synthesized following a published protocol[37] and were characterized by NMR for structure confirmation and purity. LC-MS formic acid, Chromasolv LC-MS methanol and acetonitrile were purchased from Sigma-Aldrich.

Study Design. The experiment was designed as a multicomponent pharmacokinetic study with quantitative analysis of organs (liver, kidney, heart, and brain) and biofluids (blood and urine). The aim was to explore the fate of a polyphenol microbial metabolite mixture in a mammalian system after entering the bloodstream. The experiment was divided into four time points: 15 s, 2 min, 5 min, and 15 min. Each time point was represented by four replicates. Four rats received the

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polyphenol microbial metabolite dose, whereas one blank rat served as a control without the polyphenol metabolite mixture but with an identical experimental procedure. The control animals that were not injected with the polyphenol microbial metabolites could also be considered as four control replicates to compare at each time point. The short kinetics trial time did not have an effect on the concentration and endogenous metabolite pattern in the control animals.

Twenty male rats (*Rattus norvegicus*, Wistar, Harlan Italy S.r.l.) were bred in the animal facility at the University of Trieste. The experiment was approved by the bioethical committee of the University of Trieste (doc. 865PAS12). Animal experiments were carried out in compliance with the provisions of the European Community Council Directive.[39] Rats (n = 20) at the same age (12 weeks) and weight (288 ± 20 g) were maintained in temperature-controlled rooms at 22−24 °C, 50−60% humidity, and 12 h light/dark cycles. They were fed until the night before the experiment with standard laboratory chow.

During the *in vivo* experiment, the rats were kept under quiet conditions. The cages were covered with cloth so that they were either sleeping before the anesthetic injections or were awake but without apparent nervous behavior. After receiving the anesthetic, the animals were left alone to fall asleep in another covered cage. In all cases, the animals' conditions were monitored.

Polyphenol Microbial Metabolite Mixture for Intravenous (i.v.) Administration. The polyphenol metabolite mixture comprised 23 low-molecular weight (110−228 Da) metabolites, which were selected from the key polyphenol metabolism products by gut microbiota after the consumption of berry fruits. The metabolites and dosages were selected from reports in the literature. Each metabolite was dissolved in methanol and then mixed together according to the various selected amounts.

Treatment of Rats. The rats were divided into two groups for a 2 day experiment, with two pharmacokinetic time points per day. On the first day, time points 15 and 2 min were chosen. Time points 5 min and 15 s were performed on the second day. On the night before the experiment, the rats (10 animals) were starved overnight but were provided with water *ad libitum*. They were divided into five different cages for the experiment.

The rats were anaesthetized with an intraperitoneal administration of tiletamine/zolazepam (1:1, 25 mg/kg body weight) and xylazine (10 mg/kg body weight). Each rat received an i.v. administration of 0.3 mL of PBS (phosphate buffer solution) containing 2.7 μmol of polyphenol metabolite mixture, dissolved in 30 μL of methanol. The blank control animals received an i.v. injection of 0.3 mL of PBS with 30 μL of methanol.

During anesthesia (10 min), the heart and ventilation rates were controlled. The rats were placed on their backs, and the penis was extruded by sliding the prepuce downward. With the use of a nipper, the glans penis was held at the tip. The dorsal penis vein was then visible, and at exactly 10 min after anesthesia, 0.3 mL of PBS with (treated) or without (blank) 2.7 μmol polyphenol metabolite mixture was injected using an insulin syringe. The injection site was then pressed for a few seconds, and the glans was retracted to prevent bleeding.[43] One minute before the rat was sacrificed, sodium heparin (0.1 mL, 500 IU) was injected again into the dorsal penis vein as before. For time point 15 s, the procedure was slightly different: after 10 min of anesthesia, 0.2 mL of PBS and 0.1 mL of sodium heparin with (treated) or without (blank) 2.7 μmol polyphenol metabolite mixture were simultaneously injected using an insulin syringe.

The rats were sacrificed by decapitation. Blood was drained from the body, and urine was collected through the urinary bladder with a syringe. The liver, kidney, heart, and brain were excised from the body, washed with Milli-Q water, immediately frozen in liquid nitrogen, and stored at −80 °C.

Organ Collection and Extract Preparation. *Extraction from Blood and Urine.* After sampling, an aliquot of blood (5 mL) was transferred into aqueous methanol (95:5, v/v) at a ratio of 1:9 (v/v). The internal standard *trans*-cinnamic acid-d_5 was dissolved in aqueous methanol at a concentration of 100 mg/L for monitoring the extraction protocol. The sample was shaken and extracted for 10 min. The extract was then centrifuged for 5 min at 3600 rpm at 4 °C. The blood extract with the buoyant (noncellular) portion was transferred to 50 mL dark glass vessels and stored at −80 °C.[44] The choice of extracting blood instead of plasma or serum was made to eliminate the need for a centrifugation or blood-clotting step, during which metabolic changes might have occurred *ex vivo*.[60]

In the same way, an aliquot of urine was collected with a syringe and extracted with aqueous methanol (95:5, v/v). The urine was weighed, and the amount of solvent for extraction was adjusted to a 1:9 ratio (w/v).

Tissues. Organs that were frozen in liquid nitrogen immediately after excision were stored at −80 °C. Frozen liver, kidney, heart, and brain were ground under cryogenic conditions using liquid nitrogen with a CryoMill (Retsch, Germany) and a single 25 mm i.d. steel ball (30 s with a frequency of 25/s). Tissue powder (1 g) was transferred (without thawing) into aqueous methanol (5:95 v/v) at a ratio of 1:9 (w/v). The internal standard *trans*-cinnamic acid-d_5 was dissolved in the aqueous methanol at a concentration of 100 mg/L to monitor the extraction protocol. The samples were extracted on an orbital shaker for 10 min, then centrifuged and decanted as described for blood. The final volume was then adjusted to 10 mL to balance any possible variation in the amount of water among the organs and stored at −80 °C.

Solid Phase Extraction (SPE) Purification and Sample Preparation. The SPE protocol was applied following a procedure developed by Passamonti et al.[61] with modifications as reported by Gasperotti et al.[38] The cartridges used for the SPE purification were Biotage Isolute ENV+, 1 g (Uppsala Sweden).

The internal standard butyric acid-d_7 was dissolved in methanol/water (50:50 v/v) at a concentration of 1 mg/L and added to the sample to monitor the quantitative recovery during sample reconstitution.

Targeted Metabolomics Analysis by UHPLC-ESI-MS/MS. A targeted metabolomics analysis was performed with an ultraperformance liquid chromatographic system (UHPLC) coupled to a tandem mass spectrometer. The system used was an ACQUITY UPLC system coupled to a Xevo TQ triple quadrupole via an electrospray (ESI) interface (Waters, Milford, MA, USA). The separation of the 23 target metabolites and 2 deuterated internal standards was performed with a Waters ACQUITY UPLC column and HSS T3 (100 mm × 2.1 mm, 1.8 μm) equipped with a guard column. The injection volume was 10 μL. Mobile phases of 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B) were used. Chromatographic separation was performed using a gradient as follows: 0 min, 5% B; 0−3 min, 5−20% B; 3−4.30 min, 20% B; 4.30−9 min, 20−45% B; 9−11 min, 45−100% B; 11−14 min, 100% B; and 14.01−17 min, 5% B as equilibration time. For calibration, a standard mixture of polyphenol metabolites was serially diluted in aqueous methanol (50:50) at a concentration range of 0.01 μg/L to 100 mg/L. Quantitative data were processed with Targetlynx software (Masslynx, Waters). Details of the UHPLC-ESI-MS/MS method and quantification are described in Gasperotti et al.[38]

Blood Residual Subtraction for the Brain Samples. The concentrations in the rat brain may be significantly influenced by the quantities of metabolites in the residual blood. The correction was made by estimating the amount of metabolites in the intravascular blood present in the brain, assuming that the volume of brain blood is 47.7 μL/g.[46] We then subtracted this from the total amount found in the brain. The results of this subtraction are presented in Figure 3, which also compares the data of the brain before the blood residual subtraction.

Calculation of Pharmacokinetics Parameters. Pharmacokinetic parameters were calculated using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) with a one-phase decay function suitable for i.v. injection experiments. The extrapolated dose at time 0 (t_0) and the dose at steady state (t_∞) were obtained from the plotted curves for each metabolite detected and quantified in the blood. The area under the curve ($AUC_{0\rightarrow 15min}$) and the elimination rate (k_e) were also obtained from the plotted curves.

Statistical Analysis. The quantitative data for the PMM levels are presented as the mean values ± SD (n = 4) for all of the graphs. The concentrations in the brain of the treated animals were compared against those of the controls using Student's t test, with p values < 0.1 considered to be statistically significant. Relaxing the significance threshold to 0.1 enabled us to identify the most potentially relevant information and

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minimize type II error in these experiments while involving the least number of animals, in compliance with the legislation regulating the use of animals in scientific procedures.

■ ASSOCIATED CONTENT

Supporting Information

Information regarding quality control acquisition, recovery, and data variability. Figure S1: Polyphenol microbial metabolites in urine and their distribution kinetics. Figure S2: Polyphenol microbial metabolites in kidneys and their distribution kinetics. Figure S3: Polyphenol microbial metabolites in liver and their distribution kinetics. Figure S4: Polyphenol microbial metabolites in heart and their distribution kinetics. Table S1: Quality control acquisition. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00051.

■ AUTHOR INFORMATION

Corresponding Authors

*(S.P.) Tel.: +39 0405 588 747. E-mail: spassamonti@units.it.
*(U.V.) Tel.: +39 0461 615 140. Fax: +39 0461 615 200. E-mail: urska.vrhovsek@fmach.it.

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Notes

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