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Research Article

Quantification of free and protein-bound *trans*-resveratrol metabolites and identification of *trans*-resveratrol-C/O-conjugated diglucuronides – Two novel resveratrol metabolites in human plasma

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The polyphenol *trans*-resveratrol (t-RES) is present as t-RES-3-*O*-β-D-glycoside, termed piceid, in several plant-derived foods. Although data on the metabolism and on *in vivo* effects of t-RES have been reported, quantitative data on the metabolites formed after dietary intake of t-RES or piceid are still lacking. In this study, 85.5 mg of piceid *per* 70 kg of body weight (bw) were administered to healthy volunteers in a bolus dose. t-RES metabolites formed in plasma and urine were identified and quantified by LC-MS/MS, NMR, and HPLC-DAD analysis using chemically synthesized t-RES conjugate standards. In addition, the amount of t-RES metabolites bound noncovalently to plasma proteins was determined for the first time in humans. The metabolites identified and quantified were t-RES-3-sulfate, t-RES-3,4′-disulfate, t-RES-3,5-disulfate, t-RES-3-glucuronide and t-RES-4′-glucuronide, with t-RES-sulfates being the dominant conjugates in plasma and urine. Besides these metabolites, two novel t-RES-C/O-conjugated diglucuronides have been identified and quantified in plasma and urine. Moreover, it could be shown that up to 50% of the plasma t-RES-3-sulfate, t-RES-disulfates, and the novel t-RES-C/O-diglucuronides were bound to proteins. Total recovery of the dietary administered piceid in urine ranged between 13.6 and 35.7%.

Keywords: *trans*-Piceid / *trans*-Resveratrol / *trans*-Resveratrol glucuronides / *trans*-Resveratrol plasma protein binding / *trans*-Resveratrol sulfates

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1 Introduction

Dietary *trans*-resveratrol (3,5,4′-trihydroxy-*trans*-stilbene, t-RES) is predominantly ingested with red grapes and peanuts or products thereof and has recently been demonstrated to extend the life span of mice fed a high-caloric diet [1]. Next to that, chemopreventive effects have been reported from various animal experiments in which t-RES has been administered orally [2, 3]. Results from *in vitro* studies also point to anti-inflammatory and antioxidative effects [4, 5], although its still not clear what doses would be needed to achieve those effects *in vivo*, where t-RES is metabolized into glucuronide and sulfate conjugated compounds for which the biological significance has not been described yet [6, 7]. In the past 5 years, various human studies were conducted to identify the metabolites formed after dietary intake of t-RES [8 – 12]. However, quantitative data of t-RES conjugates formed in humans are still lacking. The predominant form of t-RES in plant-derived foods is the t-RES-3-*O*-β-D-glycoside, termed piceid. In red wine, *e.g.*, concentrations of up to 50.8 mg/L can be found [13]. The aim of our here presented human study was to elucidate the metabolism of piceid after its administration to healthy volunteers. As a result, two novel, not previously described t-RES metabolites, t-RES-C/O-conjugated diglucuronides, have been identified and quantified in human plasma and urine. Moreover, we could demonstrate that up to 50% of t-RES metabolites transported in the plasma are noncovalently bound to proteins.

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Abbreviations: bw, body weight; **t-RES,** *trans*-resveratrol; **UGT,** UDP-glucuronosyltransferase

2 Materials and methods

2.1 Chemicals

The reagents used for the experiments were purchased from Sigma–Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany), and Roche Diagnostics (Penzberg, Germany). Deuterated solvents were obtained from Euriso-top (Gif sur Yvette, France). The reagents used for the protein isolation and Western blotting were acquired from BioRad (Munich, Germany), New England Biolabs (Frankfurt/Main, Germany), and BD Biosciences (Heidelberg, Germany). t-RES was purchased from Bio De Tek (Bensheim, Germany) and *trans*-piceid (97%) from Sigma–Aldrich.

2.2 Human study design

The study was performed in accordance with the basic principles defined in the Declaration of Helsinki (World Medical Association Declaration of Helsinki, Somerset West, 1996) regarding good clinical practices. Nine healthy male volunteers between 23 and 41 years of age, weighing between 57 and 91 kg (BMI ranging from 21 to 29), participated in this controlled intervention study. All of them were nonsmokers and in a good state of health, which was confirmed by a physician. The study started with a 48 h polyphenol-free diet to exclude competitive reactions by glucuronosyl- and sulfotransferases during metabolization and to washout the polyphenols still present *in vivo*. An experimental period of 48 h followed thereafter. During these 96 h of washout and experimental period, polyphenol-rich foods such as beer, wine, fruits, vegetables, juices, tea, coffee, and cocoa products were not allowed. With the exception of the four lunches, the subjects were kept in free living conditions and were asked to report all of their foods and drinks ingested in a dietary protocol. At the beginning of the experimental period, one blood and urine sample was taken from each subject by a physician as a baseline reference, followed by administration of a single oral dose of 85.5 mg piceid *per* 70 kg of body weight (bw) dissolved in 100 mL of ethanol (15%) and made-up with low-fat milk (1.5%) to a total volume of 500 mL. This amount of piceid corresponds to the content of approximately two bottles of red wine [13]. Thereafter, urine samples were taken during the time periods 2, 6, 12, 24, 48 h and blood samples were drawn 1, 2, 4, 6, 8, 10 and 24 h after administration. These time periods and time points were basically chosen according to Goldberg *et al*. [8], although the time points of 6, 8, 10 and 24 h were added to ensure plasma and urine concentrations of t-RES metabolites reached the baseline levels at the end of the study. Urine samples were stored at −20°C, blood samples were immediately separated into plasma and erythrocytes by centrifugation and stored at −80°C until further analysis.

2.3 Synthesis of resveratrol glucuronides

The t-RES-glucuronides were synthesized and subsequently purified following the procedure reported by Wenzel *et al.* [6], with the modification that one equivalent of resveratrol reacted with four (not two) equivalents of acetobromo-α-D-glucuronic acid methyl ester in anhydrous methanol. In order to elucidate the structure of the synthesized t-RES-glucuronides, LC-MS, 1-D, and 2-D NMR measurements were performed.

2.4 LC-MS and NMR spectroscopy of resveratrol-glucuronides

For the structure determination of the t-RES-glucuronides, an Aqua C18 5 µm analytical column (150 × 2 mm id, 5 µm particle size; Phenomenex, Aschaffenburg, Germany) was coupled to a mass spectrometer which consisted of a triple-quadrupole Finnigan TSQ Quantum Discovery (Thermo Electron, Bremen, Germany) operating in positive ESI mode (ESI^+). The samples were separated using the standard HPLC gradient with aqueous formic acid (0.1%, pH 2.5) and ACN as the mobile phase. ^1H, COSY, HMQC, and HMBC spectroscopies were performed on a Bruker-AV-250 spectrometer (Bruker, Bremen, Germany) at a temperature of 350 K and on a Bruker-AMX 400 spectrometer (Bruker) at room temperature (298 K). ^{13}C experiments were performed on a Bruker-AV-360 spectrometer (Bruker) at room temperature (298 K). The t-RES-glucuronides were dissolved in DMSO-d_6 and inserted into NMR-tubes (Schott, Mainz, Germany) using tetramethylsilan (TMS) as an internal standard. Evaluation of the NMR experiments was accomplished using MestReC software (Mestrelab Research, A Coruna, Spain).

2.5 Spectroscopic data

The corresponding chemical structures are displayed in Fig. 1 and the numbering of the NMR-signals refers to them given in Fig. 1. As the compounds were analyzed as a mixture due to highly similar polarity, the NMR data were collected for the mixture of both. t-RES-2-C-β-D-/4′-*O*-β-D-diglucuronide and t-RES-2-C-β-D-/4-*O*-β-D-diglucuronide; yield 30.6 mg; UV/Vis: λ_{max} 306 nm (pH 8.2); LC-MS (ESI^+): *m/z* 581 ($[M + 1]^+$); ^1H NMR (400 MHz (at 298 K)/250 MHz (at 350 K); DMSO-d_6, COSY): δ = 3.17–3.40 [t, 3H, 3 × CH, C(2‴) – (4‴)], δ = 3.28–3.51 [t, 2H, 2 × CH, C(3″) and C(4″)], δ = 3.41–3.51 [m, 1H, CH, C(5‴)], δ = 3.51–3.57 [m, 1H, CH, C(2″)], δ = 3.57–3.68 [m, 1H, CH, C(5″)], δ = 4.76 [d, 1H, *J* = 9.7 Hz, CH, C(1″)], δ = 4.99 [d, 1H, *J* = 8.0 Hz, CH, C(1‴)], δ = 6.19 [d, 1H, *J* = 2.3 Hz, CH, C(4)], δ = 6.48 [d, 1H, *J* = 2.3 Hz, CH, C(6)], δ = 6.72 [d, 1H, *J* = 16.0, CH, C(8)], δ = 6.75 [d, 2H, *J* = 8.5 Hz, 2 × CH, C(3′) and C(5′)], δ = 7.37 [d, 2H,

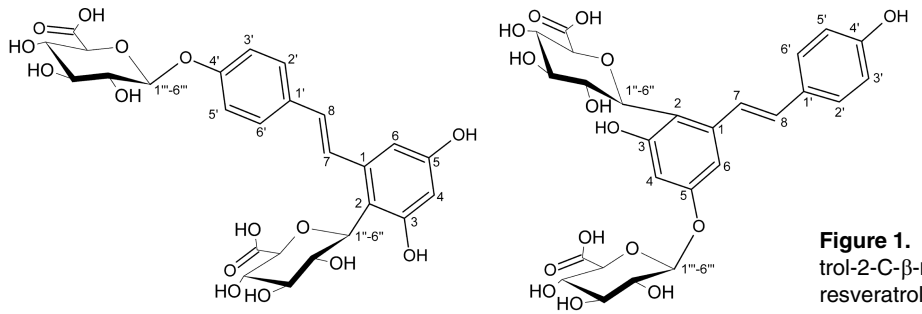

Figure 1. Chemical structure of *trans*-resveratrol-2-C-β-D-/4′-*O*-β-D-diglucuronide and *trans*-resveratrol-2-C-β-D-/4-*O*-β-D-diglucuronide.

J = 8.6, 2 × CH, C(2′) and C(6′)], δ = 7.73 [d, 1H, J = 15.8, CH, C(7)]; ^{13}C NMR (90 MHz (at 298 K); DMSO-d_6, HMBC, HMQC): δ = 72.46 [CH, C(3′) or C(4‴)], δ = 72.65 [CH, C(3′) or C(4″)], δ = 72.85 [CH, C(3′) or C(4‴)], δ = 73.05 [CH, C(3″) or C(4″)], δ = 73.44 [CH, C(2″)], δ = 75.39 [CH, C(1″)], δ = 78.91 [CH, C(2‴)], δ = 79.88 [CH, C(5‴)], δ = 80.27 [CH, C(5′)], δ = 101.04 [CH, C(4)], δ = 101.17 [CH, C(1‴)], δ = 103.86 [CH, C(6)], δ = 114.37 [C, C(2)], δ = 116.41 [2 × CH, C(3′) and C(5′)], δ = 125.15 [C, C(1)], δ = 126.37 [CH, C(7)], δ = 128.52 [2 × CH, C(2′) and C(6′)], δ = 129.10 [CH, C(8)], δ = 138.98 [C, C(1′)], δ = 79.88 [CH, C(5′)], δ = 155.91 [C, C(4′)], δ = 156.66 [C, C(5)], δ = 156.99 [C, C(3)], δ = 172.04 [COO, C(6′)], δ = 172.07 [COO, C(6″)].

For further structure elucidation, we performed an acid hydrolysis of the mixture of t-RES-C/O-diglucuronides standard compounds with 1 N hydrochloric acid over night (18 h) at 110°C, followed by a sample preparation *via* C18 cartridges (Discovery® DSC-18, 1 mL, 100 mg, Sigma–Aldrich) and LC/MS analysis. It could be shown, that the O-conjugated glucuronic acid was hydrolyzed under these conditions, which resulted in the formation of t-RES-C-monoglucuronide. The LC/MS analysis of the t-RES-C-monoglucuronide showed also representative fragmentation for C-glucosides. These results are in accordance with those reported by Sánchez-Rabaneda *et al.* [14].

2.6 Preparation of plasma and urine samples for HPLC analysis

Plasma samples were prepared following the procedure reported by Wenzel *et al.* [6]. For the preparation of the urine samples, 500 μL of urine were mixed with 200 μL ACN (100%), vortexed and centrifuged at 4°C for 5 min at 9300 × *g* using a microfuge (Eppendorf, Hamburg, Germany). The supernatant was evaporated in the dark under a nitrogen stream until dryness, redissolved in 200 μL methanol (25%) of which 90 μL were injected onto the HPLC column.

2.7 Preparation of plasma samples to determine the protein binding of resveratrol metabolites

The protein binding of t-RES and t-RES metabolites was determined following a modified method reported by Belguendouz *et al.* [15]. First, the binding of t-RES to BSA was determined by incubating aqueous solutions of BSA with t-RES (dissolved in ethanol) in a ratio of 10:1 in Pyrex® tubes (Schott) at 37°C for 24 h. Thereafter, the solutions were diluted 1:3 and 1.5 mL thereof were centrifuged with Amicon® ultracentrifugal filter devices (exclusion size 5 kDa; Millipore, Schwalbach, Germany) at 4000 × *g* for 2 h. Afterwards, the filtrates were analyzed using the HPLC-DAD method described below. The filter residues on the other hand were washed three times with 1000 μL methanol (100%) and centrifuged again at 4000 × *g* for 1 h. The three methanol filtrates were combined, evaporated in the dark under a nitrogen stream until dryness, redissolved in 500 μL methanol (25%) of which 10 μL were injected onto the HPLC column. Further on, the binding of t-RES to plasma proteins was determined by incubating mixtures containing 450 μL of reference plasma (not containing any detectable t-RES, LOD: 0.005 μg/mL), 100 μL of protease inhibitor (Roche Diagnostics) and 50 μL of a t-RES solution (16.67 mmol/L ethanol (100%)) at 37°C for 0, 2, 4 and 24 h. Thereafter, 500 μL of these incubated mixtures were diluted with 1000 μL of Millipore water and processed as described above. A portion of 500 μL of the plasma samples obtained from the study participants were diluted with 1000 μL of protease inhibitor and analyzed as described above to determine the protein binding of the t-RES metabolites. To exclude nonprotein binding of t-RES to the Amicon® ultra centrifugal filter devices, control experiments were performed with t-RES and revealed a recovery of 99%.

2.8 HPLC analysis of the plasma and urine samples

Plasma samples were analyzed following the procedure reported by Wenzel *et al.* [6]. Identification and character-

ization of the t-RES metabolites in plasma and urine samples was performed by (i) comparing the retention times of the chromatography with the retention times of the standard compounds, (ii) addition of standards to the samples, (iii) characteristic UV spectra (λ_{max} = 306 nm), and (iv) LC-MS analysis. The standard compounds monosodium-t-RES-3-monosulfate, monosodium-t-RES-4′-monosulfate, disodium-t-RES-3,4′/3,5-disulfate (mixture of two isomers with a ratio of 60:40), trisodium-t-RES-3,4′, 5-trisulfate, t-RES-3-*O*-β-D-monoglucuronide, and t-RES-4′-*O*-β-D-monoglucuronide had been synthesized earlier by Wenzel *et al.* [6]. The standard compounds t-RES-2-C-β-D-/4′-*O*-β-D-diglucuronide, and t-RES-2-C-β-D-/4-*O*-β-D-diglucuronide were synthesized in this study for the first time and were detected by HPLC as one peak due to the similar polarity of both compounds. For quantification of the t-RES metabolites, stock solutions of the standard compounds containing 100 μg/mL of Millipore water and calibration curves of five relevant concentrations for each compound were prepared. The calibration curves were characterized by regression coefficients of R^2 = 0.999 or higher. Variation coefficients of six-fold replicates were less than 5% and recovery of the t-RES metabolites in plasma and urine samples were 97 and 99%, respectively. The limits of quantitation for the t-RES conjugates in plasma and urine samples were 0.02 and 0.05 μg/mL, respectively.

2.9 Protein isolation and Western blotting of the UGT1A1 in human plasma

Diluted plasma samples were mixed with 3 × reducing SDS loading buffer (New England Biolabs) followed by a 5 min denaturation step at 95°C. Afterwards, the protein content of the samples was adjusted to 2 μg/μL. The prepared samples were loaded onto a SDS–PAGE (10% Tris-HCl SDS-Gel; BioRad), separated *via* electrophoresis and transferred to a nitrocellulose membrane (BioRad) *via* electroblotting. The protein transfer to the membranes was controlled by Ponceau staining prior to the analyses of UGT1A1 protein expression by applying a primary antibody anti-UGT1A1 (BD Biosciences). Detection was performed by using a horse radish peroxidase (HRP) conjugated goat α-rabbit IgG secondary antibody (BD Biosciences), a chemiluminescence substrate (Bio-Rad) and a chemiluminescence-sensitive quantitation system (Kodak Image Station Biostep GmbH, Jahnsdorf, Germany).

3 Results and discussion

The results demonstrated for *in vitro* studies of t-RES in isolated cells are divers, including chemopreventive, anti-aging, anti-inflammatory, antioxidant, anti-/estrogenic, antiviral, antiatherosclerotic, and neuroprotective effects [4, 5]. However, results obtained from animal experiments and human intervention trials in which t-RES has been administered in combination with a habitual diet only support the antiaging and the chemopreventive properties [1–3]. The reason for this discrepancy might be that only some of the various t-RES glucuronidated or sulfated metabolites formed *in vivo* demonstrate a distinct biologically activity. Therefore, quantitative data on the t-RES metabolites formed *in vivo* are required to further elucidate the biopotency of dietary t-RES.

The formation of t-RES in humans has been studied in various recent intervention trials. In these studies, either t-RES [8–12] or red wine [16] were administered to healthy volunteers. In all of the earlier studies [8–10, 16], the formation of t-RES glucuronides and sulfates was determined by enzymatic treatment of the plasma or urine samples with β-glucuronidase or aryl-sulfatase, leading to the formation of nonconjugated t-RES, which was finally quantified. Although the chemical synthesis of some of the t-RES conjugates such as t-RES-3-sulfate and t-RES-4′-sulfate [17] as well as t-RES-3-glucuronide and t-RES-4′-glucuronide [18] has been described, chemically synthesized and characterized reference compounds have only been used for the quantification of t-RES metabolites in one of our earlier studies in rats [6]. In that work, t-RES-mono-glucuronides and mono-, di-, and tri-sulfates were chemically synthesized and characterized by LC-MS/MS and NMR studies, and their contents in plasma, liver, kidney, urine, and feces samples were quantified with HPLC-DAD. Now, our aim was to elucidate the formation of t-RES metabolites in humans after administration of piceid, the predominant form in which t-RES is ingested with plant-derived foods such as red grapes, red wine, or peanuts. The t-RES metabolites quantified in the plasma of nine healthy male volunteers who received a single dose of 85.5 mg piceid *per* 70 kg bw were t-RES-3-sulfate, t-RES-3,4′-disulfate, t-RES-3,5-disulfate, t-RES-3-glucuronide, and t-RES-4′-glucuronide (Figs. 2 and 3). In addition, we identified and synthesized two new t-RES-C/O-conjugated-diglucuronides (Figs. 1 and 2) using the procedure reported by Wenzel *et al.* [6]. Whereas the contents of t-RES-3-sulfate reached its maximum of 29.11 ± 4.87 μg/dL (0.95 ± 0.16 μmol/L) 1 h after piceid administration, the peak concentration of t-RES-disulfates were quantified between 6 and 8 h after the dietary load, reaching absolute values of 12.72 ± 2.72 μg/dL (0.33 ± 0.07 μmol/L) for t-RES-3,4′-disulfate and 36.56 ± 6.48 μg/dL (0.94 ± 0.17 μmol/L) for t-RES-3,5-disulfate. Contents of t-RES-glucuronides also reached their maximum 6 h after ingestion, with amounts of 6.47 ± 1.50 μg/dL (0.16 ± 0.04 μmol/L) for t-RES-3-glucuronide, 7.78 ± 1.82 μg/dL (0.19 ± 0.05 μmol/L) for t-RES-4′-glucuronide and 20.19 ± 5.10 μg/dL (0.35 ± 0.09 μmol/L) for t-RES-diglucuronides. Elimination of all of these glucuronides and sulfates from the plasma was completed 24 h after piceid ingestion. Furthermore, the results clearly demonstrate that, in the plasma, t-RES-disulfates

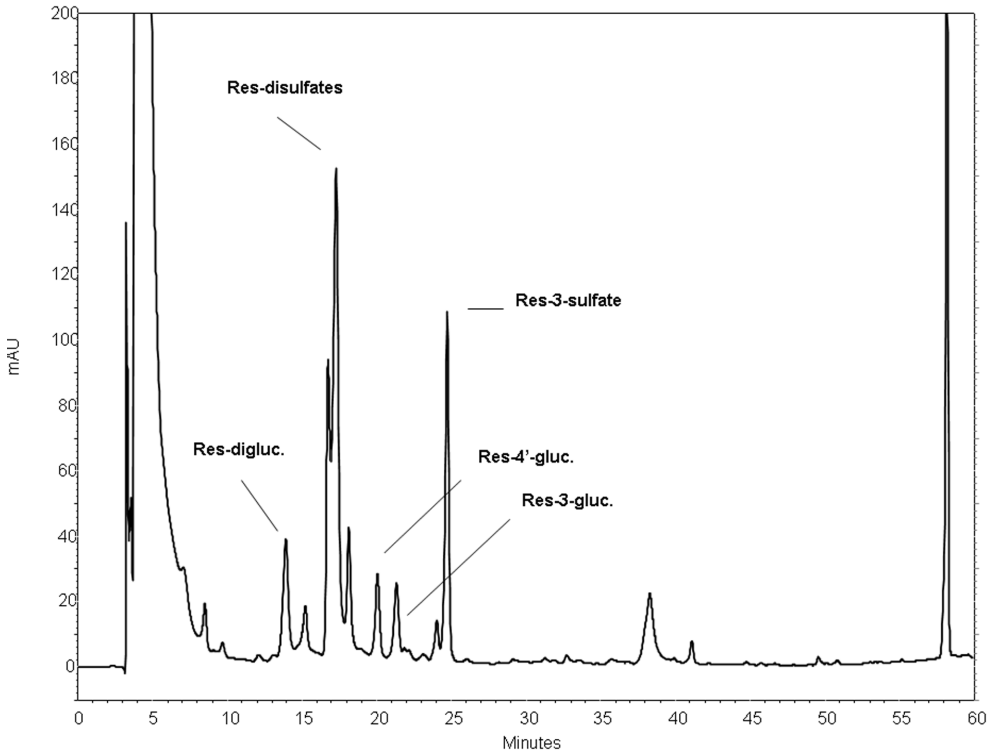

Figure 2. Representative HPLC-DAD chromatogram (λ = 300 nm) of a plasma sample 4 h after the administration of a single dose of 85.5 mg piceid *per* 70 kg bw.

are the quantitatively dominating metabolites over glucuronidated conjugates, whereas neither piceid nor t-RES aglycone were detected (LOD: 0.005 μg/mL). As this is the first time quantitative data on the formation of t-RES metabolites are reported, there are no comparable data from the literature. However, in a pharmacokinetic study, Boocock *et al.* [12] investigated the formation of t-RES metabolites in human plasma and urine after a single dietary load of 1 g. After this very high load, which is clearly not representative for the intake with a habitual diet, t-RES aglycone was detected, showing a peak concentration after about 1.5 h. In addition, different t-RES-sulfates and -glucuronides were detected and identified by LC-MS/MS fragmentation patterns, although no quantitative data were provided due to the lack of reference compounds.

The first human intervention trial in which the bioavailability of t-RES was studied was carried-out by Goldberg *et al.* [8]. In this study, a total of four healthy subjects received 25 mg t-RES *per* 70 kg bw added either to vegetable juice, white wine, or white grape juice. The peak plasma concentration of t-RES and its conjugated forms, which were determined by subtracting the amount of nonconjugated t-RES detected prior to enzymatic hydrolysis from the amount of t-RES analyzed following enzymatic hydrolysis, was observed 30 min after the dietary load, reaching a maximum concentration of approximately 2 μmol/L. One year later, Meng *et al.* [9] published data after bolus administrations of grape juice preparations containing either piceid or t-RES (0.5 – 1 mg/kg bw). Again, as the maximum plasma concentration of total t-RES (with and without enzymatic hydrolysis) were detected after 1.5 h (3.3 μmol/L). In another human intervention study on six volunteers, Walle *et al.* [10] administered a single oral dose of 25 mg ^{14}C-t-RES. One hour after administration, t-RES plasma concentrations reached about 2 μmol/L. In this study, only trace amounts of less than 5 ng/mL of t-RES aglycone were detected by counting the ^{14}C-radioactivity. As indicated by enzymatic treatment of the samples and by LC-MS fragment analysis, most of the t-RES detected in the plasma was conjugated with sulfate or glucuronic acid. Thus, our results presented in this work after a dietary administration of 85.5 mg piceid *per* 70 kg bw, corresponding to 50 mg t-RES, are in accordance with those reported by Walle *et al.* [10], although we administered piceid instead of t-RES.

Next to the quantification of t-RES glucuronides and sulfates, we were interested in the question whether these compounds could have also been bound to plasma proteins. At least for t-RES aglycone, a noncovalent binding to BSA [15], HSA [19], or plasma lipoproteins [20] has been reported from *in vitro* studies. Thus, we first determined the noncovalent protein binding of t-RES to BSA in *in vitro* experiments following a procedure reported by Belguendouz *et al.* [15]. Briefly, centrifugal filters were used to cut off the proteins (exclusion size 5 kDa) from the BSA or

Table 1. Binding of *trans*-resveratrol (t-RES) to BSA and plasma proteins (values are means of duplicates)

Sample	Incubation time (h)	t-RES recovery (μmol/L)
BSA (100 μmol/L) + t-RES (10 μmol/L)	24	
Filter		8.72
Eluate		n.d.[a)]
BSA (1000 μmol/L) + t-RES (100 μmol/L)	24	
Filter		95.1
Eluate		n.d.[a)]
Plasma (450 μL)[b)] + t-RES (690 μmol/L)	0	
Filter		650
Eluate		20
Plasma (450 μL)[b)] + t-RES (690 μmol/L)	24	
Filter		632
Eluate		7

a) n.d., not detected (LOD = 0.022 μmol/L).
b) 50 μL Plasma = 36 mg plasma proteins.

plasma samples and to degrade the bonding by adding methanol as the organic solvent. When a molar ratio of BSA (100 μmol/L): t-RES (10 μmol/L) of 10:1 was studied, 87.2% of the added t-RES was bound to the protein after 24 h of incubation (Table 1). After increasing the concentrations of BSA and t-RES by a factor of 10, it was shown that 95.1% of the added t-RES was bound noncovalently to BSA. The time dependent binding of t-RES to plasma proteins was studied in *ex vivo* model experiments using human plasma samples in which no t-RES had been detected prior to the experiments. It could be demonstrated that binding of t-RES to plasma proteins occurred rapidly after the addition of synthesized t-RES. Right after mixing, prior to any incubation, 93.6% of the t-RES added was bound to plasma proteins, whereas 2.9% were quantified as unbound t-RES. By lengthening the incubation time up to 24 h, the recovery of added t-RES was 91.0% for the protein-bound compound and 1.1% for unbound t-RES (Table 1). Based on these results, the plasma samples obtained after piceid administration were analyzed for their contents of t-RES metabolites bound to proteins. In Fig. 3, the dashed lines (charts (A) and (B)) indicate the time dependent plasma contents of t-RES-3-sulfate, t-RES-disulfates and t-RES-diglucuronides noncovalently bound to plasma proteins. The maximum plasma concentration of protein-bound t-RES-3-sulfate (15.27×3.05 μg/dL) was reached 2 h after piceid administration. In contrast, t-RES-disulfate concentrations reached a much higher level (35.86 ± 11.11 μg/dL) and peaked after 6 h. After 24 h, no protein bound t-RES-sulfates were detected. In total, 66.1% of the t-RES-3-sulfate and 56.4% of the t-RES-disulfates were analyzed in its nonprotein bound form, whereas 33.9 and 43.6% were quantified noncovalently bound to plasma proteins, respectively. Plasma concentrations of t-RES-diglucuronides reached a maximum 6 h after piceid administration (18.75 ± 5.67 μg/dL), while 46% of the total amount quantified was protein-bound and 54% were analyzed as unbound metabolites. These results confirm *in vitro* data already presented in the literature. Belguendouz *et al.* [15] reported that, after incubation of t-RES with lipoproteins ($d < 1.21$ g/mL) from porcine plasma or BSA for 1 h, the recovery of t-RES in the protein fraction was 87.6 and 69.4%, respectively. In 24 h cell culture experiments reported by Jannin *et al.* [19], it was demonstrated that nearly 100% of t-RES were bound to serum proteins present in the cell culture medium. The kind of binding has been recently identified by N' Soukpoé-Kossi *et al.* [20] whose experiments confirmed that the binding of t-RES to HSA is a nonspecific hydrogen binding *via* polar functional groups of polypeptides. However, we were able to quantify this noncovalent hydrogen binding of t-RES metabolites to human plasma proteins for the first time.

Another aspect we intended to address was whether the formation of t-RES-glucuronides is correlated with the plasma content of UGT1A1 protein, as the UDP-glucuronosyltransferase (UGT) is chiefly responsible for the glucuronidation of t-RES in enterocytes [21]. Therefore, the plasma samples were analyzed for their UGT1A1 contents by Western blotting. As a result, UGT1A1 protein plasma levels reached a maximum 4 – 6 h after piceid administration (data not shown). This result is in accordance with the plasma concentrations analyzed for t-RES-glucuronides which also showed a peak concentration after 6 h. We determined a correlation between the amount of enzyme protein and the formed t-RES glucuronides with $R^2 = 0.7946$. Although no comparable data have been reported so far, an induction of UGT1A1 protein expression by t-RES and piceid has been reported from several *in vitro* studies [21 – 23].

In addition to the plasma contents of t-RES metabolites, the urinary excretion of these compounds provides valuable biokinetic data for dietary administered piceid. The results obtained in this study are presented in Fig. 4. Chart (A) displays the urine concentrations of t-RES-3-sulfate, t-RES-3,4′-disulfate, and t-RES-3,5-disulfate, whereas chart (B) comprises the results for t-RES-3-glucuronide, t-RES-4′-glucuronide, and the t-RES-diglucuronides. The maximum urine concentration of t-RES-3-sulfate was reached in the time interval of 2 – 6 h after piceid administration (2.50 ± 0.31 mg). In contrast, the highest contents of the t-RES-disulfates in the urine samples were analyzed between 6 and 12 h after the dietary piceid load, reaching amounts of 1.11 ± 0.22 and 3.45 ± 0.62 mg for t-RES-3,4′-disulfate and t-RES-3,5-disulfate, respectively. In the same time interval, urine contents of the t-RES-glucuronides peaked and reached amounts of 1.94 ± 0.39, 0.73 ± 0.21, and 1.75 ± 0.31 mg for t-RES-3-glucuronide, t-RES-4′-glucuronide, and t-RES-diglucuronides, respectively. Urinary excretion of all of the t-RES metabolites quantified was completed

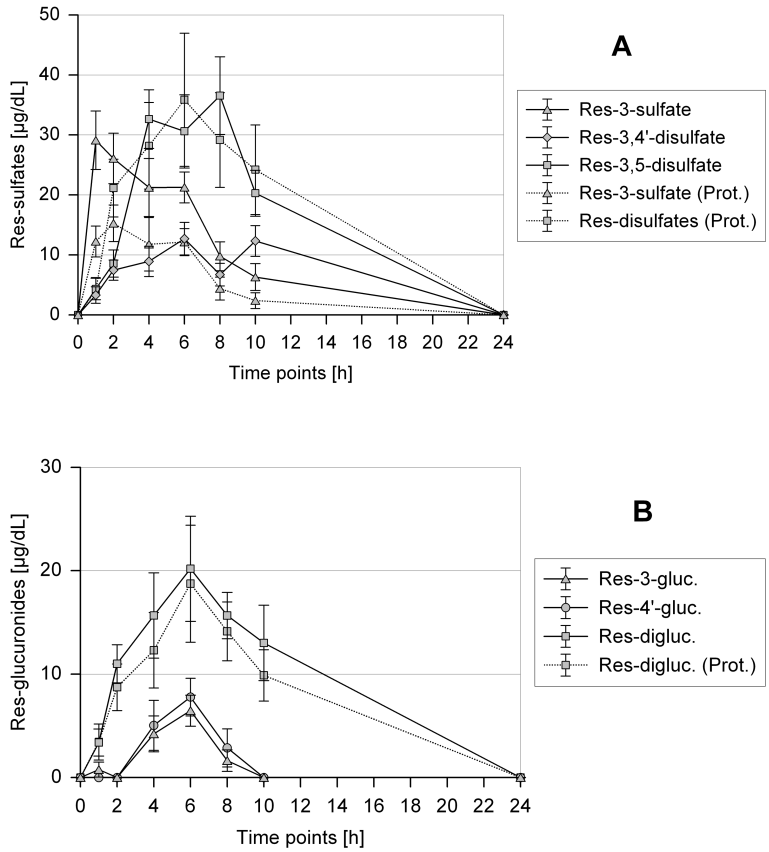

Figure 3. Plasma contents of *trans*-resveratrol conjugates after the administration of a single dose of 85.5 mg piceid *per* 70 kg bw; (A) *trans*-resveratrol-sulfates free and bound noncovalent to plasma proteins within 24 h; (B) *trans*-resveratrol-glucuronides free and bound noncovalent to plasma proteins within 24 h; Values are means ($n = 9$) ± SEM.

48 h after piceid administration. Overall, t-RES-sulfate conjugates were the quantitatively dominating metabolites, with a mean excretion of about 15% of the administered piceid dose compared to the glucuronides with a mean excretion of about 8% of the dietary piceid. Total piceid recovery in urine samples of the subjects administered 85.5 mg piceid *per* 70 kg bw ranged between 13.6 and 35.7%. Interestingly, neither piceid nor t-RES aglycone was detected in urine or plasma samples. This result indicates that piceid might be enzymatically hydrolyzed either in the small intestines or in the colon, where the intestinal microflora might provide the enzymatic activity to form the t-RES aglycone [9]. Another hypothesis is that dietary piceid might reach and enter the enterocyte in its intact form. Inside the enterocyte, the lactase phlorizin hydrolase (LPH) and the cytosolic β-glucosidase (CBG) have been demonstrated to hydrolyze piceid, resulting in the formation of t-RES [21].

In our study, the results presented for the total amount of t-RES metabolites excreted after dietary administration of 85.5 mg piceid *per* 70 kg bw are in accordance with data from earlier biokinetic studies. For example, Goldberg *et al.* [8] reported a urinary recovery of dietary administered t-RES doses of 0.03 and 1 mg/kg bw between 52 and 26%, respectively. Walle *et al.* [10] calculated the recovery of 25 mg orally administered, ^{14}C-labeled t-RES in the urine and the feces by measuring the excreted radioactivity. The recovery in the urine ranged from 53.4 to 84.9%, whereas the recovery in the feces only reached 0.3 to 38.1%. After enzymatic treatment of the samples, the amount of t-RES excreted as sulfate and glucuronide conjugates was quantified ranging from 11 to 31% and from 9 to 16%, respectively. Overall, the results from all of the human intervention studies on the biokinetics of dietary t-RES show that t-RES-sulfates are the quantitatively dominating conjugates excreted in the urine, which is in contrast to the results reported from animal studies. For example, Wenzel *et al.* [6] demonstrated that t-RES-glucuronides are the dominant metabolites in urine. These differences could be explained by a dose dependent substrate affinity of the metabolizing enzymes, the sulfotransferases and the UGT. Compared to the UGT, sulfotransferases show a smaller K_M and also a smaller V_{max} which results in a higher affinity to the substrates [24]. At increasing substrate concentrations, such as the high doses of 50 – 300 mg t-RES *per* kg bw administered to rats by Wenzel *et al.* [6], a shift of the metabolite profile from the sulfate to glucuronide conjugates might occur.

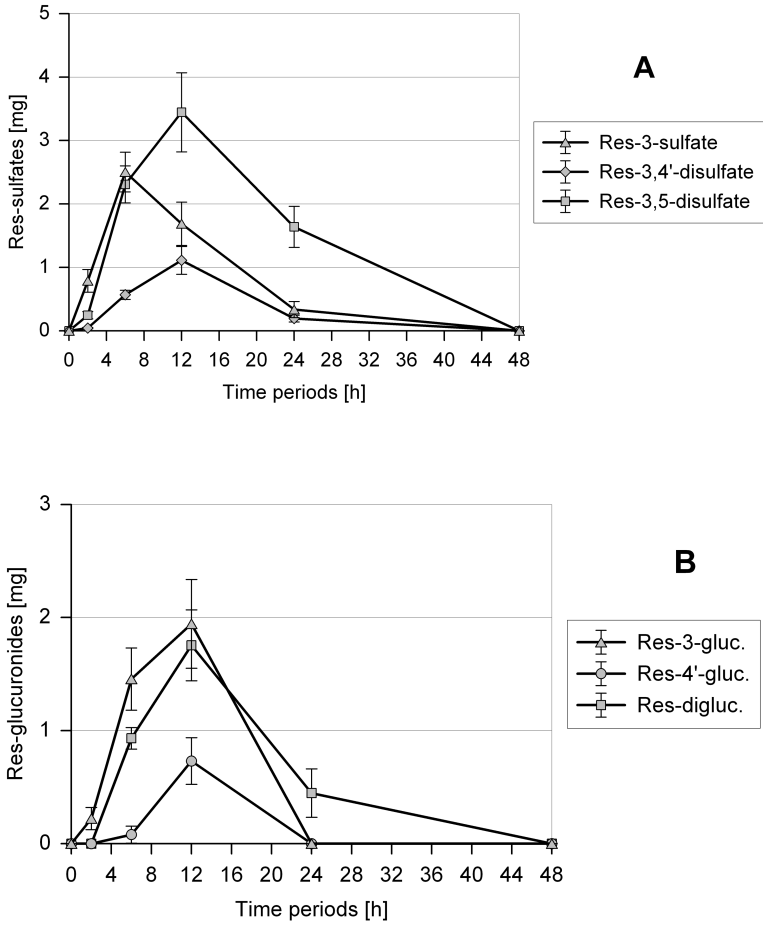

Figure 4. Urinary excretion of *trans*-resveratrol conjugates after the administration of a single dose of 85.5 mg piceid *per* 70 kg bw; (A) *trans*-resveratrol-sulfates excreted *via* the urine within 48 h; (B) *trans*-resveratrol-glucuronides excreted *via* the urine within 48 h; Values are means ($n = 9$) ± SEM.

4 Concluding remarks

In this biokinetic study, quantitative data for the plasma concentrations of protein-bound and unbound t-RES-sulfates and -glucuronides have been presented after bolus administration of piceid to healthy volunteers. Moreover, the formation of two novel t-RES-C/O-conjugated-glucuronides has been described. The biological activity of the t-RES metabolites identified herein has to be investigated in future studies. In particular, it would be of interest whether one of the t-RES metabolites is responsible for the life span extending effect reported by Baur *et al.* [1].

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