Urinary pharmacokinetics of betalains following consumption of red beet juice in healthy humans

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Accepted 28 April 2005

Abstract

The aim of the present pilot study was to characterise the renal elimination of betalains after consumption of red beet juice (RBJ). Six healthy, non-smoking female volunteers were given a single oral dose of either 500 mL of a commercial RBJ containing 362.7 mg of betalains and 500 mL of tap water, respectively, in a sequential manner. Urine was collected in intervals up to 24 h post-dose. Renal excretion of betalains was determined spectrophotometrically and quantified as betanin-equivalents. In addition, the identity of individual compounds was confirmed by HPLC coupled with diode-array detection and positive ion electrospray mass spectrometry, respectively.

The amount (mean ± S.D.) of intact betalains (betanin and isobetanin) recovered in urine was 1001 ± 273 µg corresponding to 0.28 ± 0.08% of the administered dose. Maximum excretion rates were observed after a median $t_{\text{max}}$ of 3.0 h (range 2.5–8.0 h) amounting to 91.7 ± 30.1 µg/h. The terminal elimination rate constant ($\lambda_z$) and the corresponding half-life were 0.097 ± 0.021 h$^{-1}$ and 7.43 ± 1.47 h, respectively.

Using the $\lambda_z$ estimates obtained the expected total betalain amount excreted in urine was 1228 ± 291 µg.

Based on the results obtained it is assumed that either the bioavailability of the betalains is low or that renal clearance is a minor route of systemic elimination for these compounds. The urinary excretion rates of unmetabolised betalains were fast and appeared to be monoexponential suggesting a one-compartment model. In order to get a more complete picture of the pharmacokinetics and health-promoting properties of red beet betalains, quantitative data on betalain bioavailability should include measurements of unchanged compounds and their corresponding metabolites in plasma, urine and bile.

Keywords: Betalains; Human; Pharmacokinetics; Renal excretion; Red beet

1. Introduction

Since epidemiological surveys and animal studies have shown an inverse relation between the consumption of “vegetable food” and the incidence of cancer and heart diseases [1–3], much attention has been paid to the antioxidant activity of plant pigments such as carotenoids, anthocyanins (flavonoid glycosides) and just recently the betalains [4,5]. The red–violet betacyanins (e.g., betanin and isobetanin) and the yellow betaxanthins (e.g., vulgaxanthin I and II) are water-soluble nitrogenous pigments in members of most families of the plant order Caryophyllales (except the Caryophyllaceae and Molluginaceae) and in some higher fungi [6,7]. The betalains are also important natural colorants applied for food use [8]. Among the numerous natural sources of betalains, red and yellow beet, coloured Swiss chard, grain amaranth and cactus fruits are the only foods containing these compounds [5,9,10]. Red beet (Beta vulgaris L.) is mainly consumed..
as lactofermented juice, as pickled preserves or as a cooked vegetable. Together with carrots, red beets are very frequently demanded vegetables in Germany with a per capita consumption of approximately 6.3 kg/year (second place following tomatoes) [11]. The major betalains in red beets are betanin and isobetanin together with small amounts of vulgaxanthin I and II [12]. By theory, all betacyanins and betaxanthins carrying aromatic amino compound moities are likely to stabilise radicals [5]. Both betanin and isobetanin exhibit such a phenolic and an acyclic amine group (Fig. 1), thus being excellent electron donors [13,14]. Recently, several in vitro studies on the antiradical and antioxidant activity of betalains (mainly betanin) from red beets have been published [15–18]. These results demonstrated that the betalains from red beets exert strong antiradical and antioxidant activities. Kanner et al. [15] reported inhibition of lipid peroxidation in membranes, linoleate emulsions catalysed by the “free iron” redox cycle (cytochrome c); \( \text{H}_2\text{O}_2 \)-activated metmyoglobin or lipoxygenase by red beet betanin. Due to these and more recent findings, betalains are widely considered as antioxidants protecting against oxidative stress-related disorders both in vitro and in vivo [5]. Therefore, consumers may benefit from regular consumption of products rich in betalains such as red beet juice (RBJ). However, the rate and extent of absorption, metabolism, elimination, or relative bioavailability of these bioactive compounds have been rarely considered [15].

Thus, the objective of the present study was to assess the renal elimination of betalains after oral single dose application of a commercially available red beet juice.

2. Materials and methods

2.1. Study design and procedure

Six healthy, non-smoking females were recruited with ages between 23 and 24 years and body mass indices ranging from 19.1 to 22.6 kg/m², respectively (Table 1). The study protocol was in accordance with the Helsinki Declaration of 1975, as revised in 1983, and was fully explained to all volunteers, who gave their informed written consent. Participants adhered to their usual diet and abstained from food and beverages rich in betalains or polyphenols 24 h prior to administration. Throughout the study, subjects were instructed to refrain from alcohol and medications, including over the counter drugs.

The trial was an open-label, single-centre study performed under controlled conditions following appropriate standards for human experimentation. Each subject underwent two experimental treatments, each serving as her own control by ingesting first 500 mL of RBJ (equivalent to 362.7 mg betanin) and then 500 mL of tap water. The treatments were separated by a 1-week washout phase. On the morning of the sampling days, the fasted volunteers took RBJ and tap water, respectively, together with white bread rolls and cheese. The subjects were instructed not to eat anything and to drink only tap water after RBJ consumption until lunch. Two further standardised meals (white bread rolls with cheese) were provided for lunch and dinner.

For the determination of the pharmacokinetic profile of betalains, urine samples were collected pre-dose, and quantitatively in 1-h intervals up to 5 h after RBJ ingestion (0–1, 1–2, 2–3, 3–4, 4–5 h) and then in 2-h intervals up to 11 h (5–7, 7–9, 9–11 h). A final sample was taken 24 h after dosing. The total urine volume was measured for each collection period in a graduated cylinder to the nearest 10 mL, and aliquots were stored frozen at \(-80^\circ\text{C}\) until assayed.

Table 1

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Age (years)</th>
<th>Weight (kg)</th>
<th>Height (m)</th>
<th>BMI (kg/m²)</th>
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<td>24</td>
<td>61</td>
<td>1.75</td>
<td>19.9</td>
</tr>
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<td>3</td>
<td>23</td>
<td>52</td>
<td>1.65</td>
<td>19.1</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>68</td>
<td>1.78</td>
<td>21.5</td>
</tr>
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<td>5</td>
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<td>1.68</td>
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<td>6</td>
<td>24</td>
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<td>60.0</td>
<td>1.70</td>
<td>20.8</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.32</td>
<td>7.40</td>
<td>0.07</td>
<td>1.53</td>
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</tbody>
</table>
2.2. Biostatistical methods

Solvents and reagents were purchased from VWR (Darmstadt, Germany) and were of analytical or HPLC grade. Sephadex LH-20 was from Amersham (Uppsala, Sweden) and deionised water was used throughout. Red beet juice (ALNATURA®, Bickenbach, Germany) was purchased at a local store in Jena, Germany.

2.2.1. Quantification of betalains

The eluent used was 0.05 M KH₂PO₄ : CH₃OH (85:15, v/v), protected by a LiChrospher 100 RP-18 guard column (particle size: 5 μm, 4 mm; Merck, Darmstadt, Germany) and an AS-4000 autosampler (Merck-Hitachi), a 990 diode array detector (Waters, Milford, MA), and an LC–MSD trap column (Phenomenex, Torrance, CA, USA) equipped with a security guard C₁₈ ODS (4 mm, 5 μm; Phenomenex, Torrance, CA, USA) equipped with a guard column C₁₈ ODS (5 μm, Phenomenex, Torrance, CA, USA). The mobile phases were 1% (v/v) formic acid in water (eluent A) and MeCN-water (80:20, v/v; eluent B). A gradient was followed to 33% B at 44 min, then 100% B in 6 min, before re-equilibration. In all experiments, simultaneous monitoring was performed at 280 nm for phenolics and 538 nm for betanin, respectively. The injection volume was 50 μL.

To achieve satisfactory concentration for mass spectrometric analyses, urine samples required concentration under reduced pressure at room temperature on a rotovaporator. However, high colourless phenolics contents as monitored at 280 nm precluded betacyanin identification. In such cases, further purification was necessary. Since ethyl acetate fractionation [20] was found to remove colourless phenolics insufficiently and additionally requires acidification, the acid-labile betalains were purified on a Sephadex LH-20 column (180 mm × 12 mm; i.d.) instead. Pre–equilibration with deionised water was necessary before application of the concentrated urine sample. Betacyanins were eluted with deionised water at a flow-rate of 0.9 mL/min, while phenolics remained adsorbed until eluted with 100% methanol. The so obtained aqueous betalain fraction was again evaporated to dryness under reduced pressure, redissolved in purified water and analysed by LC–MS.

2.2.2. Identification of betalains

2.2.2.1. System I: HPLC–DAD. Based on an adopted method [12], betalains in juice and urine samples were analysed on an HPLC–DAD system (Merck-Hitachi, Darmstadt, Germany) equipped with a model L-6200 pump (Merck-Hitachi), a 990 diode array detector (Waters, Eschborn, Germany), and an AS-4000 autosampler (Merck-Hitachi). Separation was achieved isocratically on a Prontosil Eurobroad RP-18 column (particle size: 5 μm, 250 mm × 4 mm; i.d.; Bischoff, Leonberg, Germany) protected by a LChromopher 100 RP-18 guard column (particle size: 5 μm, 4 mm × 4 mm; i.d.; Merck, Darmstadt, Germany). The eluent used was 0.05 M KH₂PO₄ : CH₃OH (85:15, v/v), adjusted to pH 2.75 with o-phosphoric acid. The flow rate was 1.0 mL/min, and monitoring was performed at 536 nm. Samples were centrifuged at 8400 × g for 5 min prior to injection of 100 μL aliquots from the supernatant.

2.2.2.2. System II: LC–DAD–MS/MS. Betalain separation and identification was performed as described earlier [20] using an Agilent HPLC series 1100 (Agilent, Waldbronn, Germany) equipped with ChemStation software, a degasser model G1322A, a binary gradient pump model G1312A, a thermostated autosampler model G1329/H9262, a column oven model G1316A, and a diode array detector model G1315A. This HPLC system was interfaced with a Bruker (Bremen, Germany) model Esquire 3000+ ion trap mass spectrometer fitted with an ESI source running in the positive ionisation mode (range: m/z 50–1000). Nitrogen was used as the dry gas at a flow rate of 12 L/min and a pressure of 70.0 psi. The nebuliser temperature was set to 365 °C and collision-induced dissociation spectra were obtained applying helium as the collision gas (4.1 × 10⁻² bar) at a fragmentation amplitude of 1.2 V (MS/MS).

Separation was achieved at 25 °C on a Luna 18(2)-reversed phase column (250 mm × 4.6 mm, i.d.) with a particle size of 5 μm (Phenomenex, Torrance, CA, USA) equipped with a security guard C₁₈ ODS (4 mm × 3.0 mm, i.d.). The mobile phases were 1% (v/v) formic acid in water (eluent A) and MeCN-water (80:20, v/v; eluent B). Starting with 2% B, a gradient was followed to 33% B at 44 min, then 100% B in 6 min, before re-equilibration. At a flow-rate of 0.8 mL/min, simultaneous monitoring was performed at 280 nm for phenolics and 538 nm for betanin, respectively. The injection volume was 50 μL.

To achieve satisfactory concentration for mass spectrometric analyses, urine samples required concentration under reduced pressure at room temperature on a rotovaporator. However, high colourless phenolics contents as monitored at 280 nm precluded betacyanin identification. Therefore, further purification was necessary. Since ethyl acetate fractionation [20] was found to remove colourless phenolics insufficiently and additionally requires acidification, the acid-labile betalains were purified on a Sephadex LH-20 column (180 mm × 12 mm; i.d.) instead. Pre–equilibration with deionised water was necessary before application of the concentrated urine sample. Betacyanins were eluted with deionised water at a flow-rate of 0.9 mL/min, while phenolics remained adsorbed until eluted with 100% methanol. The so obtained aqueous betalain fraction was again evaporated to dryness under reduced pressure, redissolved in purified water and analysed by LC–MS.

2.3. Pharmacokinetic evaluation

Based on the urinary concentrations of betalains (expressed as betanin-equivalents) and the scheduled times, non-compartmental pharmacokinetic evaluation was performed according to standard methods [21] using the WinNonlin Professional software (version 4.1, Pharsight Co., Mountain View, CA, USA). The following input data were applied: start and end time of each urine collection interval (Δt), urine concentrations (C), and urine volumes (V) from which the midpoint of each collection interval and the renal excretion rate for each interval (R) was computed according to Eq. (2):

\[
R = \frac{CV}{\Delta t}
\]

where Δt denotes the sampling interval.

The following pharmacokinetic parameters were subsequently derived from urinary excretion rates: the maximal renal excretion rate (Rmax), the midpoint of the respective collection interval associated with the maximal observed excretion rate (tmax), and the area under the renal excretion rate curve from time 0 to the last measured rate (AURC₀–∞).
AUCR0−tz was calculated according to the linear trapezoidal rule. The renal elimination rate constant (λz) was assessed by ln-linear regression of the terminal segment of the excretion rate versus time curve. The optimal regression fit was determined by WinNonlin using at least the three last excretion rates as the period of the highest possible coefficient of correlation. The times used for regression were manually adjusted if appropriate. The negative value of the slope of the fitted linear regression line of the unweighted data represents λz and ln (2) divided by λz denoting the terminal elimination half-life (t1/2). Using λz and the renal excretion rate at the midpoint of the last collection interval (R(t)) the expected total amounts of betacyanins excreted in urine (Ae0−∞) were extrapolated by Eq. (3).

\[ Ae_{0-\infty} = \text{AUCR}_{0-\infty} + \frac{R(t)}{\lambda_z} \]  

Moreover, the observed total amount of betanin-equivalents recovered in urine from time 0 up to 24 h (Ae0−24) was determined by multiplying the concentration with the urine volume of the respective sample in each collection interval and summing up all intervals after dosing subsequently. The fraction of orally administered betalain excreted into urine within 24 h (fR) was calculated by dividing Ae0−24 by the betalain dose administered. Values below the LOQ were set to 0.

### 2.4. Statistical evaluation

A comprehensive data summary was performed by means of descriptive statistics for all continuous target parameters (number of observations, arithmetic mean, standard deviation (S.D.), and median). For pharmacokinetic parameters with a log-normal distribution, geometric means and geometric coefficients of variation (CV%) were additionally calculated. Tests for the normality of the distribution were omitted due to small sample size.

Unless otherwise indicated, values for the estimates of pharmacokinetic parameters given in the text are the geometric means.

### 3. Results

After RBJ consumption, betanin [15S] and its C15-isomer isobetanin [15R] (Fig. 1) were excreted in the volunteers’ urine (Fig. 2B). In addition to these genuine red beet pigments, further minor components could be detected in the samples after ingestion. However, for mass spectrometric analyses, removal of co-eluting colourless phenolics was proven to be a prerequisite allowing for unambiguous pigment identification. Whereas betanin and isobetanin could be readily identified, both by their typical pseudomolecular ions (m/z 551) and their specific daughter ions (m/z 389) in the MS2 mode (data not shown), the remaining compounds could not be unambiguously assessed.

While no betalains could be detected in the volunteers’ urine after ingestion of 500 mL of tap water, the pharmacokinetic parameters of betalains (expressed as betanin-equivalents) from the six subjects under study are summarised in Table 2. The individual and mean renal excretion rates are presented in Fig. 3, according to which a mono-exponential decline of betalain excretion after reaching the
Table 2

Summary statistics and sample characteristics of urinary pharmacokinetic parameters of betalains (betanin-equivalents) following administration of a single oral dose of beetroot juice (*B. vulgaris*) to healthy volunteers

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>$R_{\text{max}}$ (mg/h)</th>
<th>$t_{\text{max}}$ (h)</th>
<th>AUC$_{\text{Rmax}}$ (mg-h)</th>
<th>AURC$_{0}$ (mg-h)</th>
<th>$A_{\text{e}0}$ (mg)</th>
<th>$f_{\text{e}}/f_{\text{e}}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>6.0</td>
<td>763.8</td>
<td>0.077</td>
<td>9.05</td>
<td>1088.6</td>
</tr>
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<td>2</td>
<td>93.5</td>
<td>3.5</td>
<td>827.9</td>
<td>0.084</td>
<td>8.28</td>
<td>1100.1</td>
</tr>
<tr>
<td>3</td>
<td>87.6</td>
<td>2.5</td>
<td>680.5</td>
<td>0.088</td>
<td>7.85</td>
<td>899.6</td>
</tr>
<tr>
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<td>111.7</td>
<td>8.0</td>
<td>1178.7</td>
<td>0.130</td>
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<td>1435.8</td>
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<tr>
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<td>57.5</td>
<td>2.5</td>
<td>568.7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>137.3</td>
<td>2.5</td>
<td>1298.6</td>
<td>0.104</td>
<td>6.64</td>
<td>1616.8</td>
</tr>
</tbody>
</table>

Geometric mean (geometric CV%): $87.5$ (34.4) $3.7$ (54.3) $849.2$ (32.8) $0.095$ (21.2) $7.30$ (21.2) $1201$ (23.9) 22.9 $0.27$ (26.4) $972.3$ (26.4) $0.27$ (26.4)

Mean ± S.D.: $91.7 ± 30.1$ $4.2 ± 2.3$ $886.6 ± 289.1$ $0.097 ± 0.021$ $7.43 ± 1.47$ $1228 ± 291$ $23.3 ± 4.7$ $1001 ± 273$ $0.28 ± 0.08$

Median: $90.6$ $3.0$ $795.9$ $0.088$ $7.85$ $1101$ $24.3$ $906.4$ $0.25$

ND: not determined (determination of $t_{1/2}$ was not possible).

The geometric mean of the maximum observed renal excretion rate ($R_{\text{max}}$) was $87.5$ µg/h with values ranging between 58 and 137 µg/h. The midpoints of the collection intervals associated with $R_{\text{max}}$ ranged between 2.5 and 8.0 h post-intake (median 3.0 h). Inter-individual variability of $R_{\text{max}}$ as characterised by the geometric CV of 34% was moderate (Table 2).

The geometric mean of the total amount of unmetabolised betanin and isobetanin (expressed as betanin-equivalents) excreted during 24 h was $972.3$ µg with values ranging between 762 and 1426 µg. This corresponds to a fraction of an orally administered betalain dose ($f_{\text{e}}/f_{\text{e}}$) of 0.27% (range 0.21–0.39%). The inter-individual variation of $f_{\text{e}}/f_{\text{e}}$ was found to be moderate with geometric CVs of about 26% (Table 2).

The geometric mean of the terminal elimination half-life ($t_{1/2}$) of betanin and isobetanin (expressed as betanin-equivalents) amounted to 7.3 h with values ranging between 5.3 and 9.1 h. Inter-individual variability in $t_{1/2}$ was moderate (geometric CV of 21.2%) (Table 2).

4. Discussion

For drug approval, a comprehensive pharmacokinetic evaluation is mandatory which requires extensive pre-clinical and clinical studies. In contrast, for functional food components, an analogous regulation demanding an in depth pharmacokinetic appraisal of specific food ingredients in humans has not yet been established. Although many definitions for "functional food" have been proposed, scientific consensus has been achieved that "a food can be regarded as functional if it is satisfactorily demonstrated to beneficially affect one or more target functions in the body, beyond adequate nutritional effects, in a way which is relevant to either an improved state of health and well-being or reduction of risk of disease" [22]. In addition, there must be a clear-cut difference to drugs which are aimed at preventing or curing diseases. Therefore, the present study is rather related to functional foods and is considered to obtain evidence of the proposed effects of betalains on human metabolism.

Our results demonstrate that betacyanins were absorbed from the gut into the systemic circulation in their intact forms. Nevertheless, the extent of absorption remains unknown as...
the bioavailability may range from less than 1 to 100%. While plasma kinetics and urinary excretion of betalains from food sources have been studied previously [15,23], exact data on oral bioavailability based on plasma concentrations have never been reported in literature. In contrast to drugs, the absorption and subsequent bioavailability also depend on the respective food source.

Betacyanins of RBJ were identified in urine immediately after ingestion supporting earlier data on renal betalain excretion. Whereas pigment contents in urine were reported to amount to 0.5–0.9% of the ingested dose via 300 mL of RBJ containing 120 mg of betanin previously [15], the amount excreted was only 0.28% in this work. Tesoriere et al. [23] found 3.7% of the ingested betanin dose after ingestion of 500 g cactus pear fruit pulp providing 16 mg betanin. This points to a possible deviation from dose-proportional absorption characteristics, but can also be explained by the different spectrophotometric assays used [19] and by inter-individual metabolic variation of the volunteers. Nevertheless, in the studies mentioned, the renal excretion of betalains in humans was apparently low and very similar to that of flavonoids [24–26]. If low renal excretion reflects primarily the low extent of absorption, then the instability of the molecules in the digestive environment, bacterial degradation in the gut, and mechanisms of absorption would play a major role [23,27].

The low renal excretion also indicates that other pathways of betalain elimination may exist such as biliary excretion, enterohepatic circulation and metabolism. Furthermore, there is increasing evidence that the principal part of absorbed flavonoids re-appear in the systemic circulation after conjugation, mainly as glucurono- and sulfon conjugates [28–32]. However, up to now, neither glucuronides nor sulphonates or methylated betalain conjugates were reported in human plasma or urine samples. Therefore, the identity of the minor metabolites from betanin and/or isobetanin as shown in Fig. 2B will shed more light on this rather speculative issue.

The remarkable inter-individual variation in the times of the maximum observed betalain excretion rates (range 2.5–8.0 h) may indicate a dependency from tubular reabsorption. If the betalains under investigation are subject to significant tubular reabsorption, then the urine flow rate may have influenced renal clearance considering that the fluid volumes ingested by the subjects (500 mL plus water ad libitum) varied remarkably.

Previous papers by Kanner et al. [15] and Tesoriere et al. [23,33] postulated a charge-related interaction of betanin with negatively charged biomolecules both in vitro and in vivo. It is also feasible that an inclusion or adsorption reaction of the whole betanin molecule had taken place for which the charges are mainly irrelevant. In addition, the respective solvent in which the binding reactions are carried out may govern the ionic charge. To point out the relevance of the medium acidity in aqueous solutions, the charge alterations of betanin and isobetanin upon pH changes [34,35] are shown in Fig. 4. In future studies, consideration of the pH-dependent ionisation of betalains may help interpret data both with respect to bioavailability issues but also considering interactions between betalains and biomolecules.

Fig. 4. Charge alteration of betanin [15S] and isobetanin [15R] in aqueous solution resulting from different pH values (modified after [34,35]). Glc: glucose.
In conclusion, the present pilot study demonstrated that the fraction of the ingested dose excreted unchanged into the urine is extremely low. This suggests that either the betalains are only slightly bioavailable or that renal clearance is a minor route of systemic elimination for these compounds. The renal excretion rates of unmetabolised betalains (betanin and isobeta- lains) present in RB1 were fast and appeared to be monoexponen- tial suggesting a one-compartment model. In order to get a more complete picture of the health-promoting properties and pharmacokinetics of red beet betalains, quantitative data on betalain bioavailability should include measurements both on unchanged compounds and their metabolites in plasma, urine and bile.

Besides betalains, red beet contains further components displaying remarkable antioxidative properties such as cyco- Dopaglucoside, hydroxycinnamic acids and their deriva- tives [36–39]. Possibly, the whole complex of “antioxi- dants” present in red beets together with their corresponding metabolites may help protect cellular systems from oxidative damage in vivo.

Acknowledgements

The authors are indebted to all volunteers who participated in this study.

References


[4] Cai Y, Sun M, Corke H. Antioxidant activity of betalains from plants displaying remarkable antioxidative properties such as cyco- Dopaglucoside, hydroxycinnamic acids and their deriv- tives [36–39]. Possibly, the whole complex of “antioxi- dants” present in red beets together with their corresponding metabolites may help protect cellular systems from oxidative damage in vivo.


