

Short Communication

Absorption of red clover isoflavones in human subjects: results from a pilot study

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In addition to soya-derived preparations, red clover-based dietary supplements have gained considerable interest as an alternative isoflavone (IF) source. While metabolism and bioavailability of the main IF from both sources have already been investigated, studies are still lacking on the biokinetic behaviour of IF, which are present in red clover in minor amounts. In the present pilot study, in which seven volunteers ingested a single dose of a commercial red clover dietary supplement, we focused on the absorption of three such IF, irilone (IRI), prunetin (PRUN) and pseudobaptigenin (PBAP). The compounds were measured as aglycones after enzymatic hydrolysis. A single intake of an amount of as low as 3.8 mg IRI (out of 38.8 mg IF in total) resulted in an IRI plasma concentration of 0.35 (SD 0.16) μM at 6.5 h post-ingestion. Compared to the plasma concentrations found for daidzein (0.39 μM) and genistein (0.06 μM), expected to be the main IF metabolites in plasma, the present findings indicate that IRI might possess a relatively high bioavailability. Furthermore, PRUN and PBAP were detected in human plasma for the first time.

Isoflavones: Irilone: Bioavailability: Red clover: Dietary supplements

Red clover-based extract preparations containing isoflavones (IF) have gained increasing importance especially for post-menopausal women as a target group. Compared to soya with daidzein (DAI), genistein (GEN) and glycitein (GLYC) as the present IF, red clover contains at least seven additional IF, with formononetin (FORM) and biochanin A (BIOA) being the dominating ones⁽¹⁾. Furthermore, irilone (IRI), prunetin (PRUN), pseudobaptigenin (PBAP), calycosin and pratensein have been detected in red clover. These IF are characterised by the presence and absence of methoxy- and/or methylenedioxy groups attached to the IF skeleton at different positions⁽¹⁾. The bioavailability and metabolism of soya IF have already been investigated^(2–4). However, only three studies in human subjects have been published dealing with the bio-transformation of the main red clover IF FORM and BIOA with the minor IF being neglected^(4–6). Howes *et al.*⁽⁶⁾ reported that a daily ingested dose of about 80 mg red clover IF for 2 weeks led to considerable total plasma levels of FORM, BIOA and their demethylated metabolites DAI and GEN, respectively, which occurred in higher concentrations than the parent compounds themselves. This can either be explained by the microbial O-demethylation of FORM and BIOA⁽⁷⁾ and a subsequent colonic absorption, or by the oxidative demethylation of FORM and BIOA catalysed

by cytochrome P450 enzymes⁽⁸⁾. The aim of the present study was to provide a more comprehensive IF profile in human plasma after intake of a single bolus dose of a commercially available red clover supplement. Special focus was given to the detection of minor IF in order to evaluate their *in vivo* importance.

Materials and methods

Design of the pilot study

Four male and three female volunteers (24–30 years of age, BMI ranging from 20.2 to 26.5 k/m^2) participated in the study and gave their appropriate consent to the study protocol. The study was conducted according to the guidelines laid down in the Declaration of Helsinki and was approved by the ethics committee of the University of Potsdam. Verbal informed consent was obtained from all subjects and formally recorded. The volunteers were considered to be healthy and had not taken any antibiotics for at least last 6 months. The red clover supplement (MenoStabil, Bad Heilbrunner Naturheilmittel GmbH&Co, Bad Heilbrunn, Germany) used was analysed by LC–UV–ESI(+)-MS following the clean-up procedure as described previously⁽⁹⁾. Two gelatine capsules

Abbreviations: BIOA, biochanin A; DAI, daidzein; FORM, formononetin; GEN, genistein; GLYC, glycitein; IF, isoflavone; IRI, irilone; PBAP, pseudobaptigenin; PRUN, prunetin.

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contained approximately 38.8 mg IF in total (calculated as aglycones). The IF content that could be measured based on the available pure standard substances was 8.7 mg BIOA, 18.9 mg FORM, 3.8 mg IRI, 1.3 mg DAI, 1.2 mg GLYC and 0.2 mg GEN.

Volunteers were asked to abstain from any kind of food items or supplements containing IF 3 d before the red clover supplementation. A list of items to be avoided was given to all participants. Two capsules, the daily dosage recommended by the manufacturer, were ingested as a single bolus dose together with a glass of water after fasting overnight and eating an IF-free breakfast. In order to contain costs for the present pilot study and to avoid unnecessary stress for the participants, blood samples were taken by venepuncture from selected volunteers before ingestion and from all volunteers only at a single point in time, i.e. 6.5 h after IF intake. This particular time point was chosen because the peak plasma concentration after oral uptake of the IF aglycones, GEN and DAI, occurs after about 6 h^(2,4). Thus, for the red clover IF aglycones, the predominant chemical form in the supplement, a similar time for the peak plasma concentration can be expected. For a sensitive detection of the IF, a blood withdrawal at 6.5 h post-ingestion was hence considered as optimal. Plasma was collected after centrifugation at 1900 g for 15 min at 4°C and was stored at -80°C until analysis.

Sample preparation

The plasma sample preparation and GC-MS/MS analysis were carried out as described earlier⁽¹⁰⁾ with slight modifications. In brief, 0.75 ml of each plasma sample was diluted with 5 ml triethylamine sulphate buffer (pH 5.0), spiked with 5 µl of an internal standard solution (GLYC dissolved in dimethylsulphoxide; 9.63 µg/ml) and sonicated for 10 min at 64°C. GLYC was not detectable in all unspiked plasma samples. For this reason, GLYC could be used as an internal standard. Solid phase extraction and cleavage of the phase II conjugates were carried out as already described⁽¹⁰⁾. After enzymatic hydrolysis, the samples were cleaned up again using solid phase extraction. The eluates were evaporated to dryness. The samples were extracted with ethyl acetate and dried under a stream of nitrogen. Before analysis using GC-MS/MS, they were derivatised with bis(trimethylsilyl)trifluoroacetamide:trimethylchlorosilane 99:1 (v/v) for 30 min at 70°C.

GC-MS/MS analysis

Isoflavonoid trimethylsilyl ether derivatives were separated and quantified by GC-MSⁿ analyses on a Thermo Finnigan system (gas chromatograph model Focus hyphenated to a Polaris Q ion trap mass detector from Thermo Electron Corporation, Bremen, Germany). GC separation was carried out on a non-polar capillary column (Varian VF-5ms, 30 m × 0.25 mm internal diameter, 0.25 µm film thickness, Varian Deutschland GmbH, Darmstadt, Germany) with the use of a helium carrier gas flow of 1.0 ml/min and a linear temperature gradient (150°C for 1 min, then 25°C/min to 230°C, hold for 3 min, then 10°C/min to 258°C, then 0.3°C/min to 265°C and hold for 5 min, then 10°C/min to 320°C, holding for 2 min). The injector port temperature was 250°C. Injections of 1 µl sample volume were made in the splitless mode. Mass spectra were obtained

by electron impact ionisation at 70 eV and an ion source temperature of 200°C. Full scan spectra (mass range 50–650 amu) were recorded. The MS² mode was applied using mass-to-charge ratios for precursor ions and daughter ion ranges, respectively: BIOA (413; 206–413), DAI (398; 199–398), FORM (340; 170–340), GEN (471; 235–471) and IRI (427; 213–427). Quantifications were carried out using GLYC as an internal standard compound as well as external calibration curves for FORM, BIOA, IRI, DAI and GEN by spiking IF-free control plasma with the respective IF. The minor red clover IF PRUN and PBAP were investigated as to their presence in the plasma samples, but were not quantified due to the lack of standard compounds with sufficient purity.

Chemicals

The chemicals used were of the highest grade available. GLYC, DAI and GEN were purchased from LC Laboratories (Woburn, MA, USA), IRI was purchased from LGC

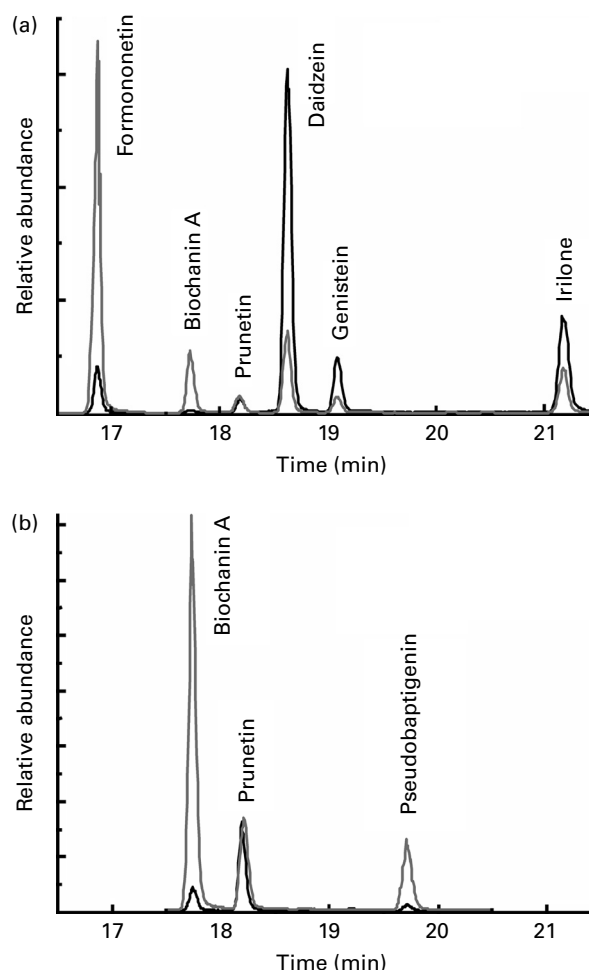


Fig. 1. Comparison of the relative isoflavone (IF) pattern of the red clover dietary supplement (—) with a representative plasma sample (---) at 6.5 h after intake of the red clover supplement. (a) Overlay of the reconstructed total ion current (TIC) chromatogram for the IF formononetin (m/z 340, 16.5–17.5 min), the structural isomers biochanin A (BIOA) and prunetin (PRUN, m/z 413, 17.5–18.3 min), daidzein (m/z 398, 18.3–19.0 min), genistein (m/z 471, 19.0–19.5 min) and irilone (m/z 427, 19.5–21.5 min). (b) Overlay of the reconstructed TIC chromatogram for BIOA and PRUN (m/z 413, 17.5–19.5 min) and pseudobaptigenin (m/z 398, 19.5–20.5 min).

Promochem (Wesel, Germany), and PBAP was purchased from APIN Chemicals (Abingdon, UK). BIOA, FORM, PRUN and dimethylsulphoxide as well as β -glucuronidase (from *Helix Pomatia*) were obtained from Sigma-Aldrich Chemical Co. (Deisenhofen, Germany). Bis(trimethylsilyl)trifluoroacetamide:trimethylchlorosilane 99:1 (v/v) was from Macherey-Nagel (Dueren, Germany). All IF standard compounds except PBAP and PRUN had a purity above 98% according to LC–UV–ESI(+)-MS analysis.

Results and discussion

Distribution pattern of isoflavones in the red clover supplement

The analysis of the hydrolysed red clover supplement showed at least nine different IF with two dominating peaks representing the main red clover IF, FORM and BIOA, as well as three smaller peaks for DAI, GEN and IRI in the MS/MS chromatogram (Fig. 1(a)). PRUN and PBAP led to less intense peaks as shown in Fig. 1(b).

Distribution pattern of isoflavones in human plasma

The plasma IF profiles, measured 6.5 h after intake of the two capsules containing 38.8 mg IF in total, were similar for all seven subjects. However, the plasma profiles were clearly different from the IF pattern observed in the red clover supplement. As expected, the two main red clover IF, FORM and BIOA, were demethylated to a great extent to form DAI and GEN, respectively. A representative chromatogram is shown in Fig. 1(a). The mean plasma concentrations for the various IF, additional information on the single volunteers' plasma levels, their body weight and the mean plasma levels for the male and female subjects are given in Table 1. The highest plasma concentrations were observed for DAI (0.385 μM) and surprisingly for IRI (0.351 μM). Additionally, a strong signal for PRUN was also observed, whereas PBAP was only detectable in small amounts. No correlations were noted between the participants' body weight and the resulting plasma level after intake of an equal amount of IF.

Interestingly, we observed higher IF plasma levels for the male volunteers, especially in the case of IRI (Table 1). However, the number of volunteers is too small to even speculate that bioavailability is higher in men.

Fig. 1(b) illustrates different absorption rates of the structural isomers BIOA and PRUN (4',5,7-trihydroxy-IF either being methylated in position C4' or C7, respectively) by showing the relative intensity of both isomers in the red clover extract as well as in a representative plasma sample, whereas BIOA is clearly more abundant in the red clover extract, and PRUN dominates in relation to BIOA in the plasma samples. Similarly for FORM, DAI and IRI (Fig. 1(a)), clear differences in the plasma pattern compared to the red clover extract pattern were also observed. These data demonstrate that the plasma concentration of a single IF cannot be assessed based on its concentration in the respective dietary supplement. Further detailed investigations on the bioconversion of all constituents have to be carried out. The need for more thorough investigations is also emphasised by the detection of PBAP in all plasma samples indicating that also minor IF are systemically available after intake of red clover dietary supplements.

The plasma levels of IRI were unexpectedly high. This observation might be explained by the results from a recent study in which we investigated the degradability of IRI compared to GEN by human faecal microbiota. We were able to show that in contrast to GEN, IRI is almost resistant to such conversion⁽¹¹⁾. Therefore, it stands to reason that a relatively high amount of the parent compound IRI is available for absorption in the intestine, especially in the colon.

Along with the results of the long-term study by Howes *et al.*, our data point out that the IF composition of a dietary supplement has a major influence on the resulting IF pattern in the plasma. Howes *et al.* used a red clover supplement with a low FORM content (FORM:BIOA ratio of 0.65). The daily IF intake of about 80 mg led to maximum DAI and GEN plasma levels of 0.25 and 0.42 μM , respectively⁽⁶⁾. Correspondingly, the present study with a single bolus intake of about 40 mg IF high in FORM (FORM/BIOA ratio of 2.17) led to plasma concentrations of the demethylated metabolites DAI and GEN of 0.39 and 0.06 μM , respectively.

Table 1. Detected isoflavone (IF) plasma levels in each of the seven subjects at 6.5 h post-intake of a single dosage of a red clover supplement (the detailed composition is given in Materials and methods) and as mean values for the female, male and entire group of participants (Mean values and standard deviations)

Subjects	Body weight (kg)	IF plasma level (nM)									
		FORM		BIOA		DAI		GEN		IRI	
Female 1	75	144		21		347		79		197	
Female 2	72	68		10		385		52		250	
Female 3	64	34		9		158		36		179	
Male 4	73	187		10		493		58		589	
Male 5	63	140		26		592		94		394	
Male 6	78	83		14		444		79		504	
Male 7	108	119		8		271		43		344	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Female		82	56	13	7	297	122	56	22	209	37
Male		132	44	15	8	450	134	68	23	458	110
Group		111	52	14	7	385	144	63	21	351	156

FORM, formononetin; BIOA, biochanin A; DAI, daidzein; GEN, genistein; IRI, irilone.

Compared to the present results, Setchell *et al.*⁽⁴⁾ reported slightly lower plasma levels for FORM, BIOA, DAI and GEN after a single bolus intake of a preparation (about 40 mg total IF) also low in FORM. However, that analysis was performed for a single subject only.

Conclusion

The present study shows for the very first time that the IF IRI is bioavailable to a high extent. IRI concentrations in commercially available red clover supplements are sufficient to lead to physiologically relevant plasma concentrations⁽¹²⁾. Along with our recent findings, we propose that the methylenedioxy bridge attached to the A ring of the IF skeleton acts as a protective group against degradation of IRI by the human microbiota. Furthermore, PRUN and PBAP could be detected in all subjects' blood plasma. So far, most of these minor IF constituents have not been toxicologically investigated. IRI and PBAP bear a methylenedioxyphenyl group, which is a structural characteristic known to exert biologically relevant effects, such as inducing hepatic cytochrome P450 enzyme expression⁽¹³⁾. Thus, further studies on the bioavailability and the metabolism of the red clover IF are advised.

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