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In vivo metabolic tracking of ^{14}C-radiolabelled isoflavones in kudzu (*Pueraria lobata*) and red clover (*Trifolium pratense*) extracts

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Absorption, distribution and elimination of ^{14}C-labelled isoflavone-containing extracts from kudzu (*Pueraria lobata*) root culture and red clover (*Trifolium pratense*) cell culture were investigated in an *in vivo* rat model. The predominant isoflavones in the kudzu extract were the glycosides puerarin, daidzin and malonyl daidzin, while in the red clover extract, the major isoflavones were formononetin and its derivatives, genistein and biochanin A, with radioactivities of 3·770 and 7·256 MBq/g, respectively. Male Sprague–Dawley rats, implanted with a jugular catheter and a subcutaneous ultrafiltrate probe, were orally administered with ^{14}C-labelled isoflavone extracts from either kudzu or clover cell cultures. Serum, interstitial fluid (ISF), urine and faeces were collected using a Culex® Automated Blood Collection System for 24 h. Analysis of bone tissues revealed that radiolabel accumulated in the femur, tibia and vertebrae at 0·04, 0·03 and 0·01 % of the administered dose, respectively, in both kudzu and red clover treatments. The liver accumulated the greatest concentration of radiolabel among the tissues tested, at 1·99 and 1·54 % of the administered kudzu and red clover extracts, respectively. Serum and ISF analysis showed that both extracts were rapidly absorbed, distributed in various tissues, and largely eliminated in the urine and faeces. Urine and faeces contained 8·53 and 9·06 % of the kudzu dose, respectively, and 3·60 and 5·64 % of the red clover dose, respectively. Serum pharmacokinetics suggest that extracts from kudzu may undergo enterohepatic circulation.

Kudzu: Red clover: Radiolabelling: Pharmacokinetics

Oestrogens are hormones that are involved in the development and maintenance of female physical traits, sexual reproduction[1], plasma lipid levels and bone mineral density[2]. Isoflavones are a class of plant-derived compounds that bind to oestrogen receptors to yield oestrogenic activity. Kudzu (*Pueraria* sp.) and red clover (*Trifolium pratense*) are leguminous species that are rich sources of isoflavones[3,4] and have demonstrated positive effects on bone health *in vitro* and *in vivo*. Kudzu-derived puerarin promoted new bone formation *in vitro* in treated rat osteoblasts[5] and *in vivo* in repair of rabbit parietal bone defects when grafted with puerarin solution mixed with collagen matrix[6]. Extracts from both *Pueraria mirifica* and *P. lobata* have also prevented bone loss in ovariectomised rats[7] and mice[8], respectively. Red clover isoflavones at a dosage of 57 mg/d have similarly exhibited potential to improve bone mineral density of the proximal radius and ulna by 4·1 % over 6 months in postmenopausal women[9]. A double-blind, randomised, placebo-controlled study of the effects of red clover isoflavones on postmenopausal women, aged 49–65 years, further supported these data, showing that women taking the isoflavone supplement experienced a reduction in bone mineral content loss and bone mineral density loss in the lumbar region of the spine[10].

While our understanding of kudzu and red clover phytochemicals has greatly improved in recent years, little is known about how these dietary constituents are absorbed, distributed, metabolised and excreted in the human body[11,12]. This is attributed to the difficulty in examining the phytochemical absorption within a complex diet and discriminating between newly absorbed phytochemicals and previously existing levels in the body[11,13]. One approach to studying phytochemical bioavailability, while circumventing these problematic issues, is the use of stable isotope- or radioisotope-labelled compounds[11], which offers a means of tracking an administered dose of phytochemicals in human subjects or animal models[12]. A key advantage of using radiolabelled compounds over non-radioactive compounds in such studies is that sensitive detection systems make it possible to administer diet-relevant doses and still be able to track pharmacokinetics and biodistribution[14].

The present study was conducted to investigate the absorption, distribution and elimination of ^{14}C-labelled isoflavone-containing extracts from kudzu root culture and red clover cell culture in an *in vivo* rat model.

Abbreviation: ISF, interstitial fluid.

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Materials and methods

Chemicals and reagents

Standards for puerarin, daidzein, genistein, formononetin and biochanin A were purchased from Sigma-Aldrich (St Louis, MO, USA) for isoflavone identification and quantification. Standards for daidzin, genistin and malonyl-genistin were purchased from LC Laboratories (Woburn, MA, USA). Standards for ononin (formononetin 7-*O*-β-D-glucoside) and sissotrin (biochanin A 7-*O*-β-D-glucoside) were purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ, USA). Isoflavones that could not be authenticated by standards due to commercial unavailability were instead quantified by a method of molecular-weight adjustment of related standards[15].

Plant material

Kudzu (*P. lobata*) seed was obtained in October 2003 from the Binkley Rd population in Johnston City (Williamson County, IL, USA). This kudzu accession was identified by Terry Esker (Natural Heritage Biologist, Illinois Department of Natural Resources) and a voucher specimen (voucher no. 227615) was deposited in the Illinois Natural History Survey herbarium. Late-flowering red clover (*T. pratense* 'Mammoth') seed was obtained from Johnny's Selected Seeds (Winslow, ME, USA).

Kudzu seeds were surface scarified with a scalpel and agitated in tap water containing two drops of polyoxyethylene sorbitan monolaurate (Tween-20; Sigma-Aldrich, St Louis, MO, USA) per litre for 14 h to overcome seed dormancy and promote germination. Seeds were surface disinfested in 0·9 % sodium hypochlorite for 15–30 min. Seeds were rinsed three times with sterile distilled water and explanted to test-tubes containing 15 ml 1/2 × Murashige and Skoog basal media (2·2 g/l)[16] with rose vitamins[17], myoinositol (0·1 g/l; PhytoTechnology Laboratories, Shawnee Mission, KS, USA), sucrose (30 g/l), agar (6 g/l; Sigma-Aldrich, St Louis, MO, USA) and pH adjusted to 5·7.

Following germination, roots were excised and transferred to 250 ml flasks containing 40 ml root culture media, which consisted of Murashige and Skoog basal media (4·3 g/l) with rose vitamins, myoinositol (0·1 g/l), sucrose (30 g/l), 1-naphthaleneacetic acid (1·0 mg/l)[18] and pH adjusted to 5·7. Cultures were maintained in the dark on a rotary shaker (150 rpm) at 25°C and subcultured at 21 d intervals by replacing all but 5 ml of the spent medium with 35 ml fresh root culture medium. Root cultures were divided approximately every 9 weeks, after they had accumulated about 25 % new growth.

Red clover seeds were surface disinfested by wrapping in filter paper, submerging in 70 % ethanol for 1 min, and then in a 0·9 % sodium hypochlorite solution with two drops of polyoxyethylene sorbitan monolaurate (Tween-20) per litre for 10 min. Seeds were rinsed five times with sterile water and explanted to 1/2 × Murashige and Skoog basal medium with rose vitamins, myoinositol (0·1 g/l), bacteriological-grade agar (6 g/l) and pH adjusted to 5·7. Petiole segments from 21-d-old seedlings were placed onto 15 ml solid red clover callus-inducing medium[19], consisting of Gamborg B5 salts (3·08 g/l)[20], B_5 vitamins, myoinositol (0·1 g/l), sucrose (20 g/l), 2,4-dichlorophenoxyacetic acid (2·25 mg/l), 1-naphthaleneacetic acid (2·0 mg/l), and kinetin (2·12 mg/l; Sigma-Aldrich, St Louis, MO, USA) and bacteriological-grade agar (6 g/l) in culture tubes. After callus cultures had developed, the undifferentiated cells were transferred to 125 ml flasks containing 80 ml callus induction medium, without agar added. Cells maintained as suspension cultures in the dark were transferred to fresh callus media (3 ml settled cell volume in 7 ml spent medium) every 14 d.

Radiolabelling

Uniformly labelled, crystalline [^{14}C]sucrose with a specific activity of 374 MBq/mmol (ICN Biomedicals, Inc., Irvine, CA, USA) was utilised as the label source. A stock solution of [^{14}C]sucrose was prepared in sterile, pH 5·7-adjusted, double-distilled water and filter-sterilised. Concentrated media, at 80 and 90 % of the final volume (32 and 72 ml per 250 ml flask for kudzu and red clover, respectively), containing all constituents except [^{14}C]sucrose, were prepared and autoclaved. Following transfer of established cultures to fresh, concentrated medium as described above, 8 ml stock [^{14}C]sucrose solution were added to kudzu root or red clover cell suspension cultures to bring the final media volumes up to 40 and 80 ml, respectively. The final concentration of [^{14}C]sucrose added was 0·248 and 0·126 MBq/ml medium for kudzu root cultures and red clover cell cultures, respectively. Culture flasks were then placed into an enclosed Plexiglas labelling chamber, designed to siphon and capture respired $^{14}CO_2$[12]. Kudzu root cultures were grown in this chamber for 21 d, before being harvested. Red clover cultures were permitted to grow for 11 d, before being removed and treated with 40 μl of a 50 μM-$CuCl_2$ solution, as an elicitor for isoflavone synthesis[21]. Red clover cultures were returned to the labelling chamber and were exposed to the $CuCl_2$ elicitor for 3 d, then harvested.

Extraction and chemical analysis of isoflavone extracts

At harvest, root and cell suspension cultures were vacuum filtered using a Buchner funnel with Whatman no. 4 filter paper. Cells and roots were rinsed with distilled, deionised water to wash away residual media and [^{14}C]sucrose that was not taken up. Excess moisture was removed by suction and fresh weight was recorded. Fresh roots or cells were then placed into a blender with 80 % methanol in a 1:5 (w/v) ratio and blended for 2 min. The methanolic extract was separated by filtration with Whatman no. 4 filter paper and stored in a flask at −20°C. The extraction procedure was repeated twice using the same volume of solvent, with the third extraction held overnight at 4°C, before filtration. The three extractions were combined, filtered with Whatman no. 1 filter paper, concentrated by rotoevaporation (Buchi Rotavapor; Büchi Labortechnik, Flawil, Switzerland) and dried down completely by lyophilisation in a freeze dryer (Labconco Freezone 4.5; Labconco Corp., Kansas City, MO, USA).

Characterisation of isoflavones in extracts

An LCQ Deca XP mass spectrometer (150–1000 mass:charge ratio (*m/z*)) attached to a photodiode array detector (UV: 262 mm) (Thermo Finnigan Corp., San Jose, CA, USA) was utilised in HPLC–electrospray ionisation–MS analysis on a C_{18}-reversed-phase column (2·1 × 150 mm, VYDAC, catalogue

no. 201 SPS215). The mobile phase was composed of 95 % double-distilled water with 5 % acetonitrile and 0·1 % trifluoroacetic acid (solvent A) and 5 % double-distilled water with 95 % acetonitrile and 0·1 % trifluoroacetic acid (solvent B). A step gradient of 0, 70, 100 and 0 % of solvent B was used at 0, 30, 35 and 40 min, respectively. Samples were prepared by dissolving approximately 20 mg dry extract in 4 ml 80 % methanol (5 mg/ml), sonicating and filtering through a 0·45 μm nylon syringe filter (Fisher Scientific, Pittsburgh, PA, USA). A quantity of 5 μl of each sample was injected for analysis at a flow rate of 200 μl/min. Commercial standards were also used in this analysis to verify UV absorption, retention time and molecular weights of isoflavones present in extract.

Surgical protocol

Male Sprague–Dawley rats (body weight 275–350 g), acclimatised to a 12 h photoperiod, were anaesthetised with 3–5 % isofluorane and delivered with O_2 at 2–4 litres/min. A jugular catheter was inserted into the right jugular vein and an ultrafiltrate probe (Bioanalytical Systems, Inc., West Lafayette, IN, USA) was implanted subcutaneously down the dorsal midline. Following surgery, the rats were administered 0·3 ml of 10 % buprenex in 0·9 % NaCl (w/v) and connected to a Culex® Automated Blood Sampling System (Bioanalytical Systems, Inc.) for blood, interstitial fluid (ISF), urine and faeces collection. Rats were given 48 h for recovery. Food (AIN93M; Dyets, Inc., Bethlehem, PA, USA) and water were provided *ad libitum* and the catheter was flushed with 10 units heparin/ml saline (0·9 % NaCl, w/v) every 15 min. All experimental procedures involving animals were approved by the Purdue Animal Care and Use Committee, which adheres to policies set forth by the US Department of Agriculture and the US Public Health Service.

Oral administration protocol

Preliminary experiments demonstrated that a dose of 0·148 MBq of ^{14}C-labelled extract could be traced effectively *in vivo* using a scintillation counter and were used as a basis for choosing dose sizes for radiolabelled kudzu and red clover extracts. Rats were food-deprived for 8 h before oral administration and 200 μl blood were drawn as the baseline. Approximately 100 mg/kg body weight of ^{14}C-labelled kudzu extract (3·770 MBq/g) or 75 mg/kg body weight of red clover extract (7·256 MBq/g) in 1 ml distilled water were administered to the rats (six rats for kudzu treatment and four rats for the red clover treatments) by oral administration, followed by an immediate rinse of the oral administration needle with 0·5 ml distilled water. The Culex® system (Bioanalytical Systems, Inc.) automatically extracted approximately 200 μl blood at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 480, 600, 720 and 1440 min after oral administration and collected ISF at 60 min intervals for a total of 24 h. Urine and faeces were collected for 24 h. Food was returned 240 min after oral administration.

Rat sample collection

Rats were anaesthetised 24 h after oral administration with approximately 0·3 ml ketamine (100 mg/ml) and xylazine (100 mg/ml). Blood was drawn from the jugular catheter until no more blood could be collected. This blood was transferred to heparinised microcentrifuge tubes, centrifuged at 5000 ***g*** for 10 min, and plasma was collected for analysis. An incision was made through the left jugular vein and the rat was perfused through the right jugular catheter with 240 ml chilled saline, which was sufficient to produce clear perfusate that exited the incision in the left jugular vein. The stomach, small intestines, large intestines, brain, heart, lungs, liver, kidneys and testes were placed into scintillation vials. The contents of the stomach, small intestines and large intestines were collected by flushing the respective organs through with 10 ml saline (0·9 % NaCl, w/v). Femurs, tibias and vertebrae were carefully removed, wrapped in saline-saturated gauze and placed into scintillation vials. All samples were stored at −80°C until ready for analysis.

Rat sample analysis

Tissue samples were prepared by macerating with a homogeniser (Bio-homogenizer model M133/1281-0; Biospec Products, Inc., Bautlesville, OK, USA). Tissue homogenates were weighed and transferred to scintillation vials in duplicate. Bones were cleaned, dried at 50°C and ground to a fine powder with a mortar and pestle. Weighed samples of ground bone were placed into scintillation vials in duplicate and dissolved in concentrated nitric acid overnight to achieve uniformity. Faeces were dried in an oven at 50°C, 20 ml of 50 % methanol were added to extract ^{14}C-labelled compounds overnight and the methanolic extract was transferred to scintillation vials. Gastrointestinal contents were prepared by adding 10 ml methanol to each sample and the methanolic extract was transferred to scintillation vials. Serum, plasma, ISF and urine required no additional preparation for analysis.

After all homogenised tissues, dissolved bones, bodily fluids and methanolic extracts of faeces and gastrointestinal contents were placed into scintillation vials in duplicate, 20 ml Ecolite scintillation cocktail (MP Biomedicals, Solon, OH, USA) were added. Samples were allowed to sit overnight in the dark, before analysis with a Beckman LS 6500 scintillation counter (Beckman Coulter, Inc., Fullerton, CA, USA).

ISF was collected at an average rate of 60 μl/h. The ultrafiltrate probe tubing was 48 inches (about 122 cm) in length, with a total internal volume of approximately 12 μl. This internal volume presents a delay between the time ISF is collected and the time ISF reaches the sample collection vial. To account for this delay and to more accurately reflect the amount of ^{14}C label present in the ISF at any given time, the ISF collection time points illustrated in Figs. 1 and 2 were shifted 12 min earlier than actually collected. Total serum and ISF volumes were approximated based on the body weight of each rat[22] and were used to estimate the percentage of ^{14}C-labelled compounds remaining in each respective fluid 24 h after oral administration.

Results and discussion

The predominant isoflavones in the kudzu and red clover extracts were unambiguously identified by HPLC–electrospray ionisation–MS and HPLC photodiode array detector using retention time, molecular weight and comparison with

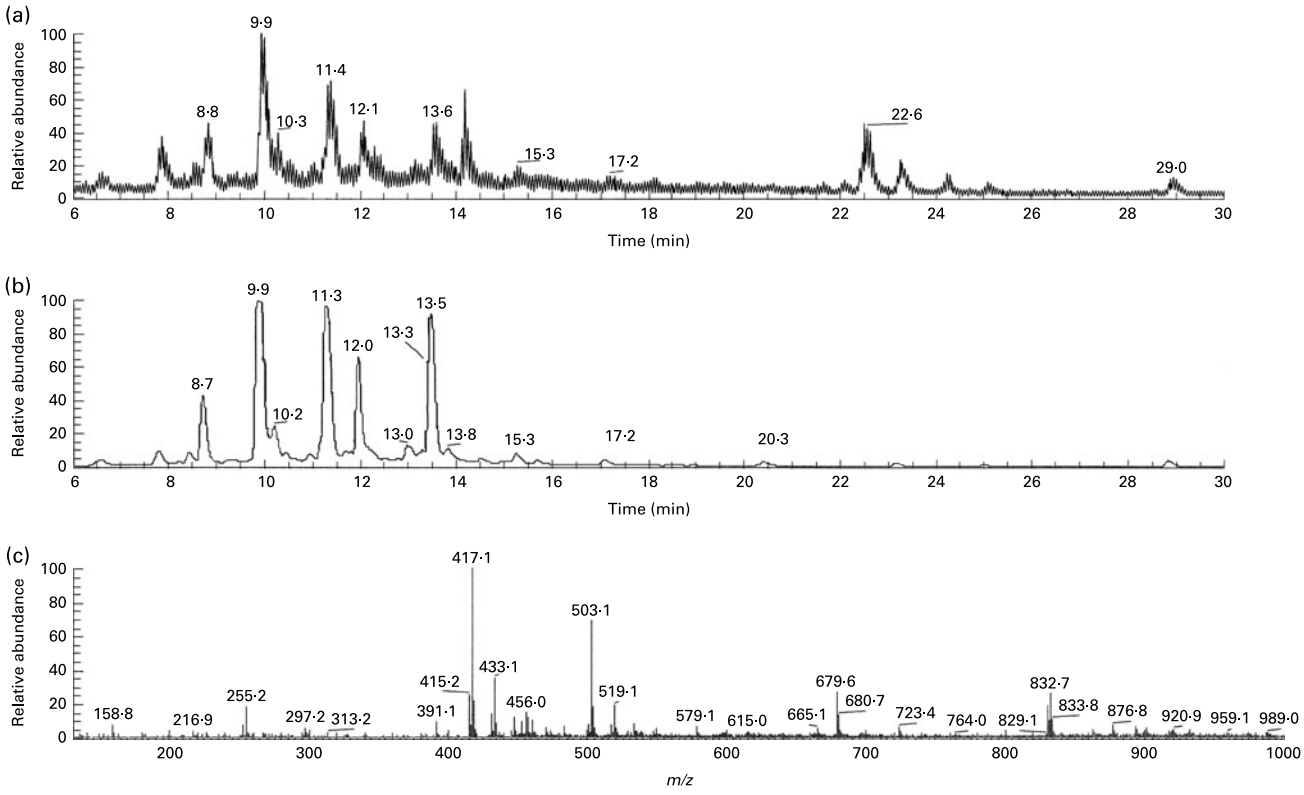

Fig. 1. Representative LC–MS spectra showing total ion current (a), LC–UV chromatogram (UV 262 nm) (b) and MS spectrum (c) of isoflavone extract from kudzu (*Pueraria lobata*) root cultures. *m/z*, Mass:charge ratio.

known commercial isoflavone standards. Kudzu isoflavones were confirmed as the glycosides puerarin, daidzin and malonyl daidzin (Fig. 1 and Table 1)[23,24]. In the red clover extract, the major isoflavones were formononetin and its derivatives, genistein and biochanin A (Fig. 2 and Table 2)[25]. Since [^{14}C]sucrose was used as the radiolabel source, ^{14}C was incorporated into all biosynthesised phytochemicals. The kudzu and red clover extracts had average radioactivities of 3·770 and 7·256 MBq/g, respectively, with standardised isoflavones composing 19·7 and 20·3 % of the total extracts, respectively.

Analysis of baseline serum, ISF, urine and faeces by scintillation counter established a naturally occurring, background level of ^{14}C in each animal, which was subtracted from levels measured after oral administration. Serum analysis showed that kudzu and red clover isoflavone extracts were rapidly absorbed, with peak serum concentrations of ^{14}C attained 15–30 min after oral administration, at an average of 0·30 and 0·33 % of the total administered ^{14}C dose per ml serum in kudzu and red clover treatments, respectively (Figs. 3 and 4). Labelled compounds from both extracts were also rapidly distributed, as seen in the extracellular fluid, collected by the ultrafiltrate probe. A second peak in serum concentration approximately 1 h after oral administration of kudzu extract may be attributed to the varied rates of absorption and distribution of different phytochemicals *in vivo*, such that one compound may already be undergoing distribution into tissues or elimination from the body, while another is still being absorbed. A plateau in serum concentration approximately 4 h (Fig. 3) after oral administration of the kudzu extract is consistent with the kudzu isoflavones puerarin[26], daidzein[27] and genistein[28] undergoing enterohepatic circulation, while also being eliminated from the body. Plasma collected 24 h after oral administration showed no difference in ^{14}C activity compared with serum collected 24 h after oral administration. Peak ISF concentrations of 0·15 and 0·23 % of the administered dose per ml were attained 1–2 h after oral administration of kudzu and red clover extracts, respectively (Figs. 3 and 4). Analysis of serum and ISF over the course of 24 h showed that kudzu and red clover phytochemicals were rapidly metabolised and eliminated. Excrement contained the greatest amount of the

Table 1. Isoflavones in radiolabelled kudzu (*Pueraria lobata*) root culture extracts detected by LC–MS analysis*

t_R (min)	Major ion $[M + H^+]$ (*m/z*)	Product ions (*m/z*)	Compound name
8·7	433		Hydroxy-puerarin
9·9	417		Puerarin
11·3	417	255	Daidzin
12·0	503	447, 255	Unassigned
13·3	433	271	Genistin
13·5	503	255	Malonyl daidzin
15·3	519	271	Malonyl genistin
17·2	255		Daidzein
20·3	271		Genistein

t_R, retention time; *m/z*, mass:charge ratio.
* See Fig. 1 for LC–MS spectra of kudzu root culture extract. The data in Table 1 correspond with Fig. 1.

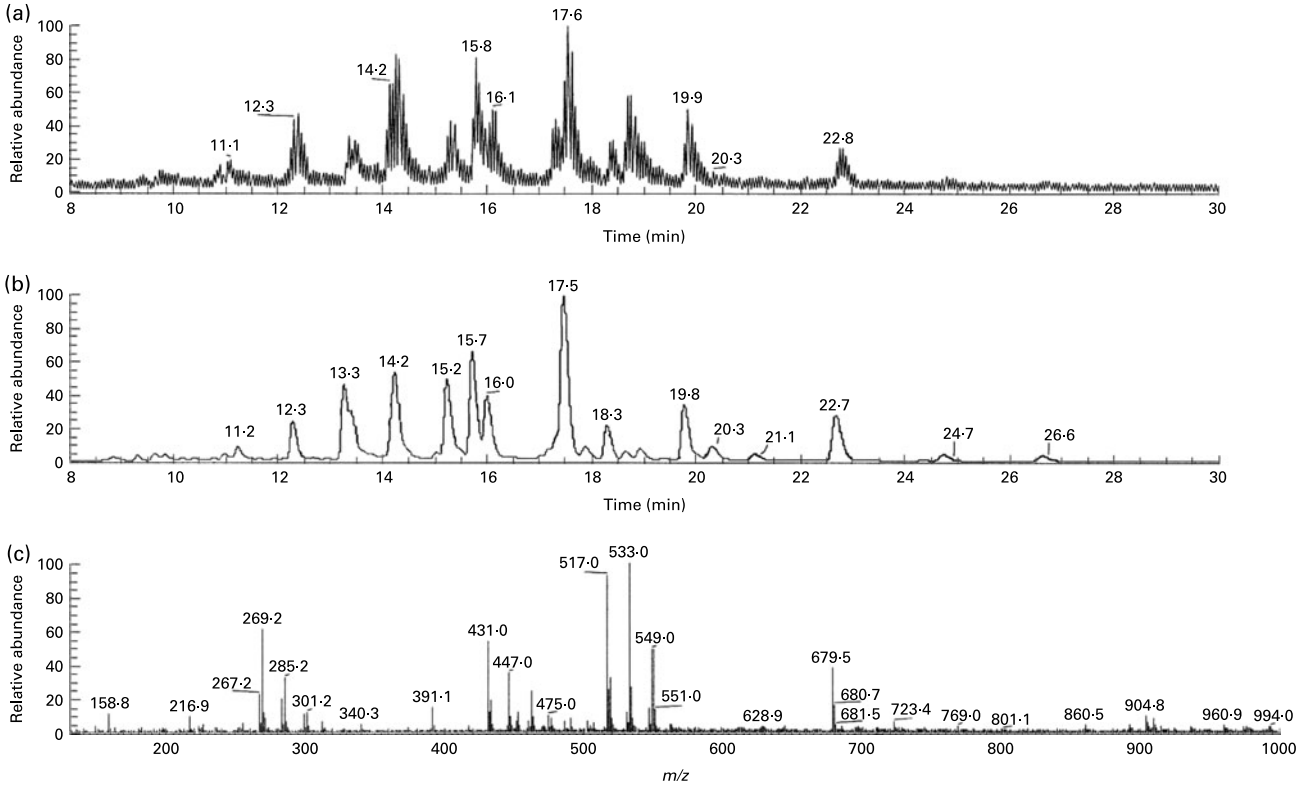

Fig. 2. Representative LC–MS spectra showing total ion current (a), LC–UV chromatogram (UV 262 nm) (b) and MS spectrum (c) of isoflavone extract from red clover (*Trifolium pratense*) cell cultures. *m/z*, Mass:charge ratio.

administered dose at 9·06 and 8·53 % in the urine and faeces, respectively, in the kudzu treatment (Table 3). While this was also true in the red clover treatment, only 4·44 and 3·59 % of the administered red clover dose were accounted for in the urine and faeces (Table 3). Total ^{14}C in all analysed bones, tissues, excrement and bodily fluids did not fully account for the total administered dose and this indicated that ^{14}C label was incorporated into other tissues such as muscle and adipose, and/or metabolism and elimination of labelled compounds in the form of exhaled $^{14}CO_2$.

For isoflavones to have an effect on bone health, they must reach the target tissues. ^{14}C-labelled compounds were detected in the bones and tissues 24 h after oral administration. In animals orally administered with either kudzu or red clover extracts, radiolabel accumulated in femurs, tibias and vertebrae, at 0·04, 0·03 and 0·01 % of the administered dose, respectively (Table 3). Successful delivery of ^{14}C-labelled kudzu and red clover extract to the bones supports the *in vitro* and *in vivo* studies that have demonstrated increased bone formation[5,6] and reduced bone loss[7,29] upon treatment with isoflavones.

Table 2. Isoflavones in radiolabelled red clover (*Trifolium pratense*) cell culture extracts detected by LC–MS analysis*

t_R (min)	Major ion [M + H^+] (*m/z*)	Product ions (*m/z*)	Compound name
11·2	417	255	Daidzin
12·3	447	285	Glycitin
13·3	433	271	Genistin
14·2	533	285	Calycosin glycosyl-malonate
15·2	519	271	Malonyl genistin
15·7	431	269	Ononin
16·0	549	431, 301	Pratensein glycosyl-malonate
17·5	517	269	Formononetin glycosyl-malonate
18·3	447	285	Sissotrin
19·8	533	285	Biochanin A glycosyl-malonate
20·3	271	285	Genistein
21·1	301	–	Pratensein
22·7	269	–	Formononetin
24·7	299	–	Irilone
26·6	285	–	Biochanin A

t_R, retention time; *m/z*, mass:charge ratio.
*See Fig. 2 for LC–MS spectra of red clover cell culture extract. The data in Table 2 correspond with Fig. 2.

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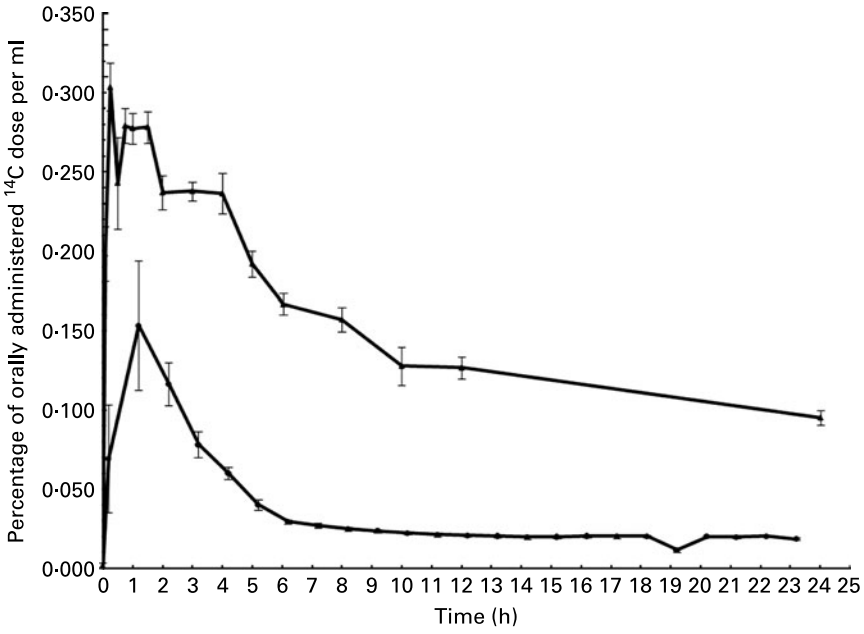

Fig. 3. Distribution of ^{14}C label for kudzu (*Pueraria lobata*) isoflavone extract in serum (–▲–) and interstitial fluid (–●–) of rats during 24 h after administration. Values are means, with standard errors represented by vertical bars.

The liver accumulated the greatest quantity of radiolabel among tissues analysed, at 1·99 and 1·54 % of the total dose for kudzu and red clover treatments, respectively (Table 1). These data are consistent with the fact that compounds which are absorbed by the digestive system are first transported to the liver before reaching the rest of the body, a phenomenon also known as the first-pass effect[28]. These observations further support the notion that isoflavones are absorbed from the intestine, travel to the liver via the portal vein, are enzymically conjugated and secreted in the bile, and may be hydrolysed by gut microflora and reabsorbed[30].

Analysis of gastrointestinal tract contents indicated that only about 0·12 % of the orally administered dose remained in the stomach, 24 h after oral administration. Larger quantities of extract remained in the small and large intestines at 1·33 and 2·66 % of the administered kudzu dose and at 0·51 and 0·99 % of the administered red clover dose, respectively.

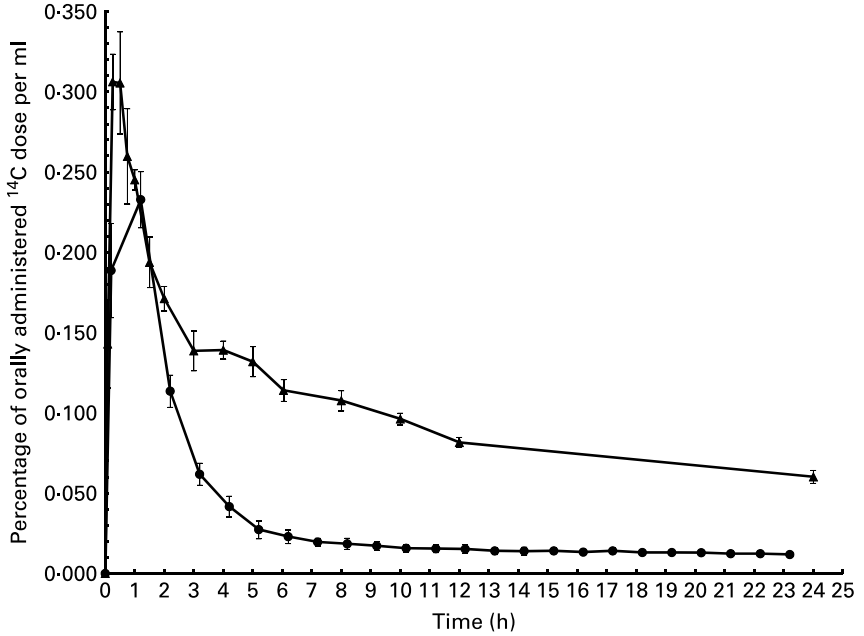

Fig. 4. Distribution of ^{14}C label for red clover (*Trifolium pratense*) isoflavone extract in serum (–▲–) and interstitial fluid (–●–) of rats during 24 h after administration. Values are means, with standard errors represented by vertical bars.

Table 3. Analysis of ^{14}C-labelled extract distribution in rats 24 h after oral administration by scintillation counter (percentage of the orally administered dose)

	Treatment	
Tissue or sample	Kudzu (*Pueraria lobata*)	Red clover (*Trifolium pratense*)
Vertebra	0·01	0·01
Left femur	0·04	0·04
Left tibia	0·03	0·03
Brain	0·00	0·00
Kidney	0·21	0·16
Liver	1·99	1·54
Lung	0·07	0·07
Heart	0·04	0·04
Testes	0·19	0·22
Stomach	0·12	0·12
Small intestine	0·67	0·68
Large intestine	0·25	0·20
Stomach contents	0·10	0·10
Small-intestinal contents	1·33	0·51
Large-intestinal contents	2·66	0·99
Faeces	8·53	3·60
Urine	9·06	5·64
Plasma	1·13	0·61
Interstitial fluid	0·49	0·55

While radioactivities of the intestinal contents were lower in the small intestines than the large, the small-intestinal tissues contained a greater quantity of radiolabel than the large intestines in both treatments. The other major tissues examined include the lungs, kidneys, heart and testes, which accumulated 0·07, 0·21, 0·04 and 0·19 % of the dose, respectively, in the kudzu treatment and 0·07, 0·16, 0·04 and 0·22 %, respectively, in the red clover treatment (Table 3).

A comparison of the areas under the serum concentration time curves shows that on average, a dose of 100 mg kudzu extract/kg body weight yielded an exposure of 6·69 % of dose × h per ml, while a dose of 75 mg red clover extract/kg body weight yielded an exposure of 4·22 % of dose × h per ml. These values will be used to establish suitable kudzu and red clover extract doses in future pharmacokinetic studies. Future studies will also include analysis for ^{14}C-labelled metabolites to better understand the biochemical mechanisms by which these phytochemicals exert an effect.

Conclusions

Biosynthesised ^{14}C-labelled isoflavones were administered to rats to determine the absorption and distribution in an *in vivo* model. These radiolabelled compounds were detected in various rat organ tissues. The results of the present metabolic tracking study support the hypothesis that isoflavones may play a role in bone health by demonstrating that radiolabelled kudzu- and red clover-derived extracts were capable of reaching and accumulating in bone tissues.

Acknowledgements

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J. G. M., M. D. G. and A. R. were students who conducted these experiments; G. G. Y., R. B. R. and P. J. L. were senior research associates who aided in the analysis; E. M. J., C. M. W. and M. A. L. were faculty members who supervised the research and took charge of the outcomes.

There are no conflicts of interest associated with this paper.

References

1. Jacobs MN & Lewis DFV (2002) Steroid hormone receptors and dietary ligands: a selected review. *Proc Nutr Soc* **61**, 105–122.
2. Genant H, Lucas J, Weiss S, *et al.* (1997) Low-dose esterified estrogen therapy: effects on bone, plasma estradiol concentrations, endometrium, and lipid levels. *Arch Intern Med* **157**, 2609–2615.
3. Kaufman PB, Duke JA, Brielmann H, *et al.* (1997) A comparative survey of leguminous plants as sources of the isoflavones, genistein and daidzein: implications for human nutrition and health. *J Altern Complement Med* **3**, 7–12.
4. Swinny EE & Ryan KG (2005) Red clover *Trifolium pratense* L. phytoestrogens: UV-B radiation increases isoflavone yield, and postharvest drying methods change the glucoside conjugate profiles. *J Agric Food Chem* **53**, 8273–8278.
5. Zhang Y, Zeng X, Zhang L, *et al.* (2007) Stimulatory effect of puerarin on bone formation through activation of PI3K/Akt pathway in rat calvaria osteoblasts. *Planta Med* **73**, 341–347.
6. Wong R & Rabie B (2007) Effect of puerarin on bone formation. *Osteoarthritis Cartilage* **15**, 894–899.
7. Urasopon N, Hamada Y, Cherdshewasart W, *et al.* (2008) Preventive effects of *Pueraria mirifica* on bone loss in ovariectomized rats. *Maturitas* **59**, 137–148.
8. Wang X, Wu J, Chiba H, *et al.* (2003) Puerariae radix prevents bone loss in ovariectomized mice. *J Bone Miner Metab* **21**, 268–275.
9. Clifton-Bligh PB, Baber RJ, Fulcher GR, *et al.* (2001) The effect of isoflavones extracted from red clover (Rimostil) on lipid and bone metabolism. *Menopause* **8**, 259–265.
10. Atkinson C, Compston JE, Day NE, *et al.* (2004) The effects of phytoestrogen isoflavones on bone density in women: a double-blind, randomized, placebo-controlled trial. *Am J Clin Nutr* **79**, 326–333.
11. Grusak MA (1997) Intrinsic stable isotope labeling of plants for nutritional investigations in humans. *J Nutr Biochem* **8**, 164–171.
12. Grusak MA, Rogers RB, Yousef GG, *et al.* (2004) An enclosed-chamber labeling system for the safe ^{14}C-enrichment of phytochemicals in plant cell suspension cultures. *In Vitro Cell Dev Biol Plant* **40**, 80–85.
13. Yousef GG, Seigler DS, Grusak MA, *et al.* (2004) Biosynthesis and characterization of ^{14}C-enriched flavonoid fractions from plant cell suspension cultures. *J Agric Food Chem* **52**, 1138–1145.
14. Lila MA, Yousef GG, Jiang Y, *et al.* (2005) Sorting out bioactivity in flavonoid mixtures. *J Nutr* **135**, 1231–1235.
15. Chandra A, Rana J & Li Y (2001) Separation, identification, quantification, and method validation of anthocyanins in botanical supplement raw materials by HPLC and HPLC–MS. *J Agric Food Chem* **49**, 3515–3521.

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16. Murashige T & Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* **15**, 473–497.
17. Rogers RB & Smith MAL (1992) Consequences of *in vitro* and *ex vitro* root initiation for miniature rose production. *J Hort Sci* **67**, 535–540.
18. Hakamatsuka T, Ebizuka Y & Sankawa U (1994) *Pueraria lobata* (kudzu vine): *in vitro* culture and the production of isoflavonoids. vol. 28, *Biotechnology in Agriculture and Forestry, Medicinal and Aromatic Plants VII*, pp. 386–400 [YPS Bajai, editor]. Berlin, Germany: Springer-Verlag.
19. Beach KH & Smith RR (1979) Plant regeneration from callus of red and crimson clover. *Plant Sci Lett* **16**, 231–237.
20. Gamborg OL, Miller RA & Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp Cell Res* **50**, 151–158.
21. Engelmann N, Rogers RB, Vattem P, *et al.* (2006) Copper chloride elicitation of *in vitro* red clover isoflavones. *J Soc In Vitro Biol* **42**, 34A.
22. Fernandez LA, Rettori O & Mejia RH (1966) Correlation between body fluid volumes and body weight in the rat. *Am J Physiol* **210**, 877–879.
23. Reppert A, Yousef GG, Rogers RB, *et al.* (2008) Isolation of radiolabeled isoflavones from kudzu (*Pueraria lobata*) root cultures. *J Agric Food Chem* **56**, 7860–7865.
24. Prasain JK, Reppert A, Jones K, *et al.* (2007) Identification of isoflavone glycosides in *Pueraria lobata* cultures by tandem mass spectrometry. *Phytochem Anal* **18**, 50–59.
25. Engelmann NJ, Reppert A, Yousef G, *et al.* (2009) *In vitro* production of radiolabeled red clover (*Trifolium pratense*) isoflavones. *Plant Cell Tissue Organ Cult* (epublication ahead of print version 26 May 2009).
26. Prasain JK, Peng N, Moore R, *et al.* (2009) Tissue distribution of puerarin and its conjugated metabolites in rats assessed by liquid chromatography–tandem mass spectrometry. *Phytomedicine* **16**, 65–71.
27. Janning P, Schuhmacher US, Upmeier A, *et al.* (2000) Toxicokinetics of the phytoestrogen daidzein in female DA/Han rats. *Arch Toxicol* **74**, 421–430.
28. Sfakianos J, Coward L, Kirk M, *et al.* (1997) Intestinal uptake and biliary excretion of the isoflavone genistein in rats. *J Nutr* **127**, 1260–1268.
29. Occhiuto F, De Pasquale R, Guglielmo G, *et al.* (2007) Effects of phytoestrogenic isoflavones from red clover (*Trifolium pratense* L.) on experimental osteoporosis. *Phytother Res* **21**, 130–134.
30. Prasain JK, Jones K, Brissie N, *et al.* (2004) Identification of puerarin and its metabolites in rats by liquid chromatography–tandem mass spectrometry. *J Agric Food Chem* **52**, 3708–3712.

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