Standardization, Screening and Clinical Evaluation of Estrogenic Isoflavones in Red Clover (*Trifolium pratense*) for Women's Health

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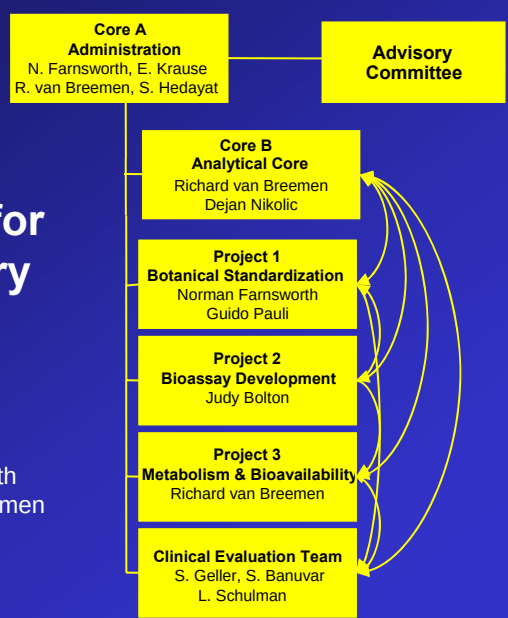


Steps Required Prior to Clinical Assessment of Botanical Dietary Supplements

1. Acquire plant material
   - Verify identity; taxonomic/microscopic/PCR
   - Record geographical origin of field specimens or point of origin of cultivated material
   - Check for pesticides; herbicides; heavy metals
   - Check microbial content
2. Establish/select appropriate bioassays
3. Bioassay several types of extracts *in vitro* and *in vivo.*
4. Identify active constituents
   - Bioassay-guided isolation or ultrafiltration LC-MS
   - Chemical characterization (MS, NMR, etc.)

Steps Required Prior to Clinical Studies

5. Use appropriate analytical method(s) such as TLC, HPLC, GC, GC-MS, LC-MS, etc., to standardize the botanical product.
6. Carry out biological standardization.
7. Stability studies are required for the standardized product
8. Pharmacologic studies are required for the standardized product
   - Metabolism (including interactions with cytochrome P450 enzymes)
   - Pharmacokinetics
   - Toxicity
   - Mechanism of Action

Risks of Estrogen Replacement Therapy Using Equine Estrogens

- Hormone replacement therapy (HRT) in menopausal women is associated with increased risks of certain cancers, stroke and dementia.*
- The leading HRT products Premarin™ and Prempro™ contain equine estrogens such as equilenin.
- Equilenin is metabolized to the toxic metabolite 4-hydroxyequilenin that is oxidized to quinoids that alkylate biopolymers and promote oxidative stress through redox cycling.

* Shumaker *et al*. **2003** *JAMA*, 2651-2662; Wassertheil-Smoller *et al*. **2003** *JAMA* 2673-2684

Alternatives to HRT: Screening Botanicals for Estrogens

- Botanical specimens were obtained by field collection, cultivation or from suppliers.
- Organic and aqueous extracts of each plant were prepared.
- Extracts were screened using ultrafiltration LC-MS for ligands to estrogen receptor (ER)-α and ER-β.
- Ligands to ER-α and ER-β were identified in *Trifolium pratense* L. (red clover) and *Humulus lupulus* (hops) but not in black cohosh (*Cimicifuga racemosa*).
- Black cohosh was determined to have serotoninergic activity.

Production and Standardization

- Red clover was grown at the University of Illinois Pharmacognosy Field Station (Downers Grove, IL) and identified by a taxonomist.
- Voucher specimens were prepared.
- Chemical standardization was carried out using HPLC-UV and LC-MS.
- PCR analysis was carried out using *Trifolium pratense* and related species.
- Extraction and hydrolysis of red clover for the clinical trial were carried out at PureWorld Botanicals (S. Hackensack, NJ) under GMP.

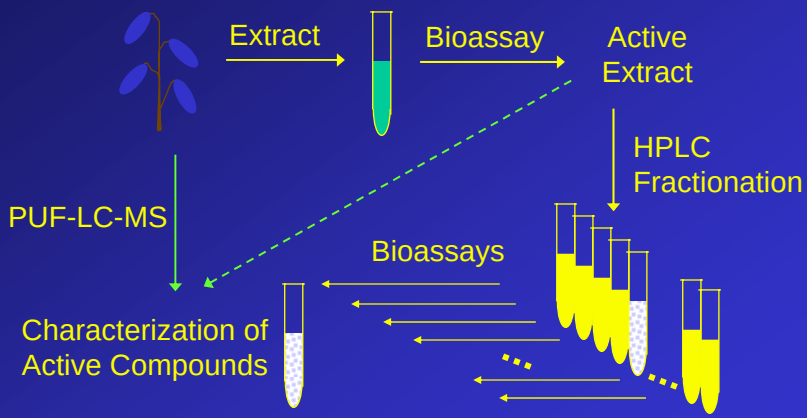
Production and Standardization

- The extract intended for clinical use was analyzed for heavy metals, herbicides and pesticides
- Microbial content was tested to be within acceptable limits
- The clinical extract was standardized biologically by Project 2.
- Chemical standardization was carried out and was set to a total of 15% isoflavones (by weight).

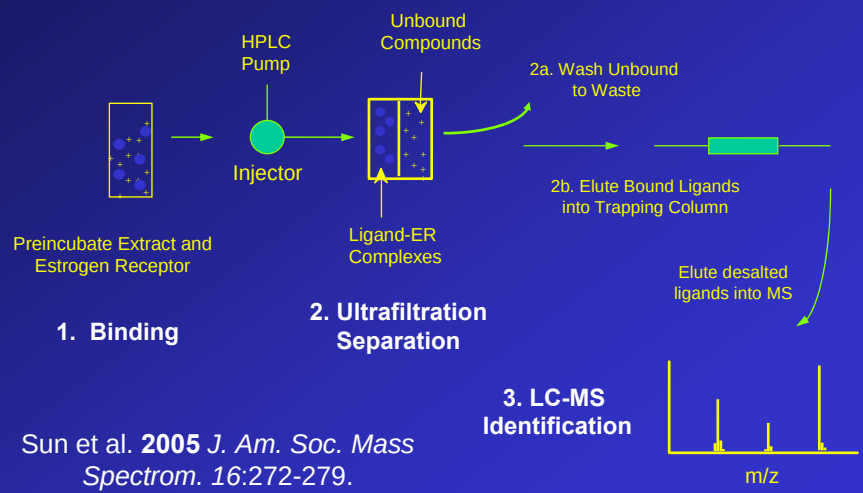
Ultrafiltration LC-MS Screening of *Trifolium pratense* L. (Red clover) for Estrogens

Natural Product Drug Discovery

PUF-LC-MS vs Bioassay Guided Fractionation

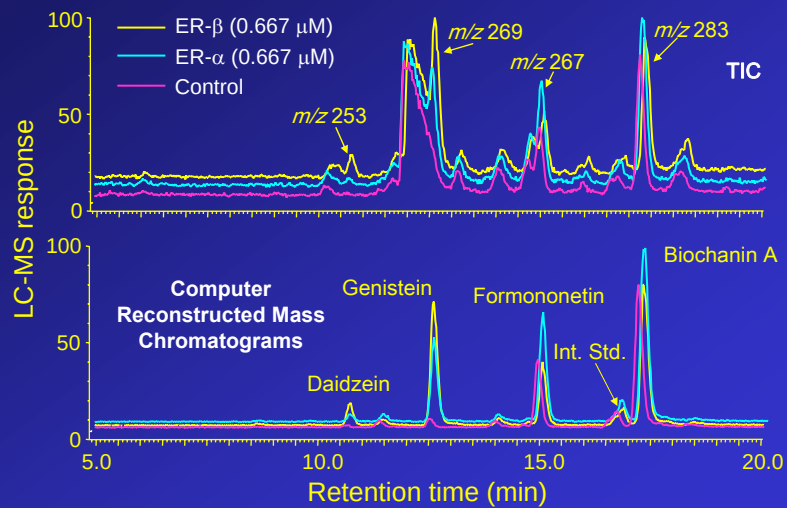


Pulsed Ultrafiltration-Mass Spectrometric Screening for Ligands of ER-α and ER-β



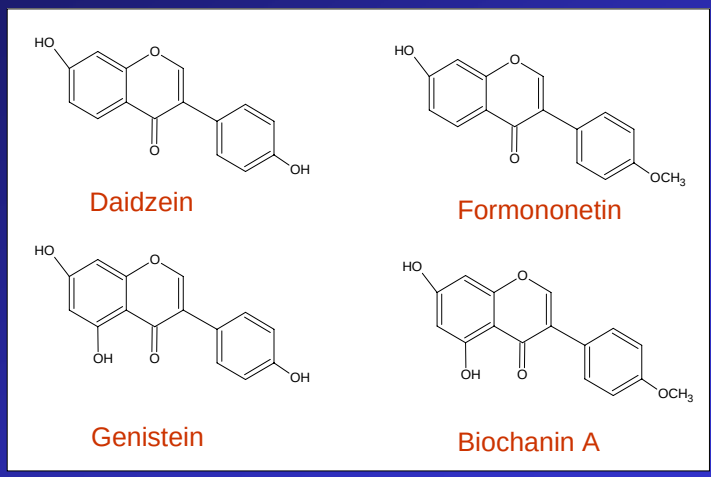
Sun et al. **2005** *J. Am. Soc. Mass Spectrom. 16*:272-279.

Ultrafiltration LC-MS Screening of *Trifolium pratense* L. (Red Clover) Extract (10 mg/L)



Liu, et al. **2001** *J. Agric. Food Chem. 49*:2472.

Estrogenic Isoflavones in Red Clover Used for Chemical Standardization of the Dietary Supplement for the Clinical Trials



Biological Standardization and Mechanism of Action

Extracts were assayed for estrogenicity

- ER-α and ER-β
  - Competitive binding assays using [^{3}H]-estrodiol and recombinant estrogen receptors
- Induction of alkaline phosphatase activity and up-regulation of progesterone receptor mRNA in Ishikawa (endometrial) cells
  - Assays of estrogenicity and anti-estrogenicity
- Estrogenic properties in ovariectomized rats
  - Uterotrophic effects were confirmed *in vivo.*

Estrogenic Activities of Red Clover Standardized Extract and Pure Isoflavones

	ER-α IC_{50} µg/mL or µM	ER-β IC_{50} µg/mL or µM	AP Induction EC_{50} µg/mL or µM	PR mRNA Fold Induction
E2	0.021	0.015	0.00014	47
DMSO	N/A	N/A	N/A	1.0
Red Clover	18	2.0	1.9	30
Daidzein	17	1.2	0.53	2.0
Formononetin	104	60	N/A	1.9
Biochanin A	35	4.1	4.6	1.4
Genistein	0.3	0.02	0.33	3.0

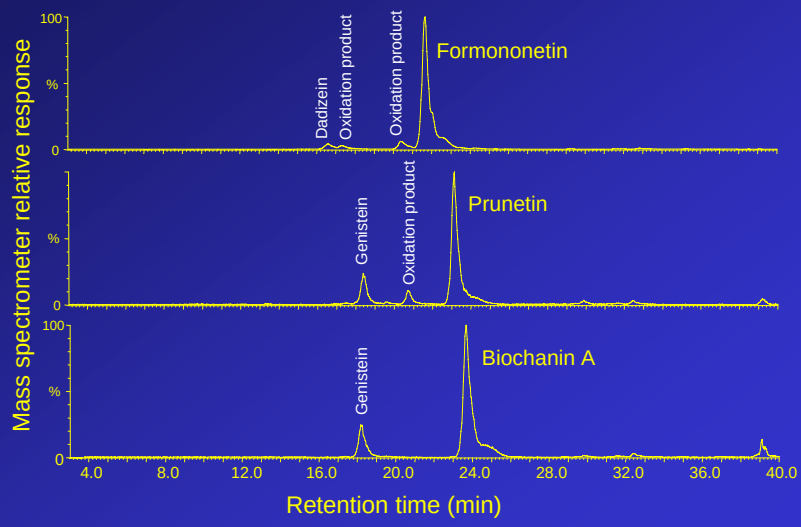
Metabolism, Bioavailability and Toxicity Screening

- No toxic compounds or electrophilic metabolites were found in vitro or in vivo for the standardized red clover extract.
- In comparison, electrophilic and potentially toxic metabolites of equine estrogens contained in Prempro have been reported such as 4-hydroxy-equilenin.
- No heavy metal or pesticide contaminants were detected in the standardized botanical extracts.

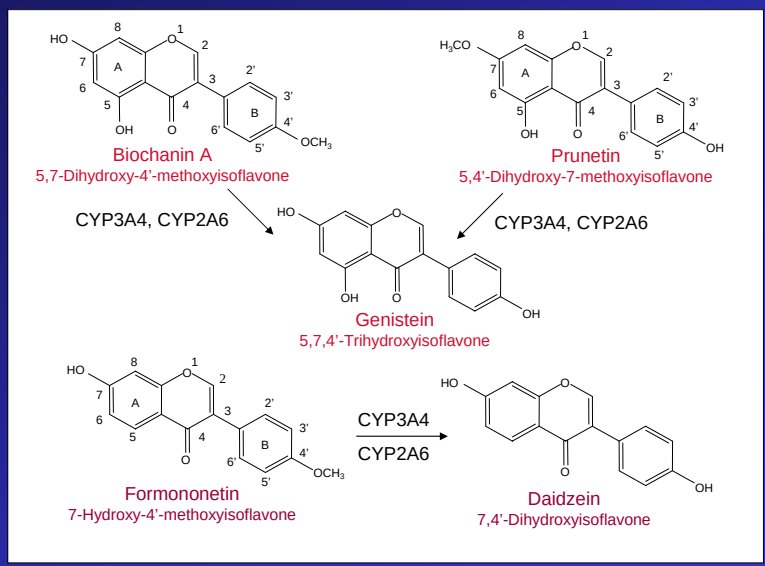
Preclinical Studies of Metabolism, Toxicity and Intestinal Absorption

- Ultrafiltration tandem mass spectrometry and human liver microsomes were used to screen the red clover extract for reactive metabolites. None were detected.
- Human hepatocytes and liver microsomes were used to generate isoflavone phase I and II metabolites which were identified using LC-MS-MS.
- The cytochrome P450 ezymes responsible for phase I O-demethylation were identified.
- The intestinal permeability of the red clover isoflavones was investigated using human intestinal epithelial Caco-2 cells

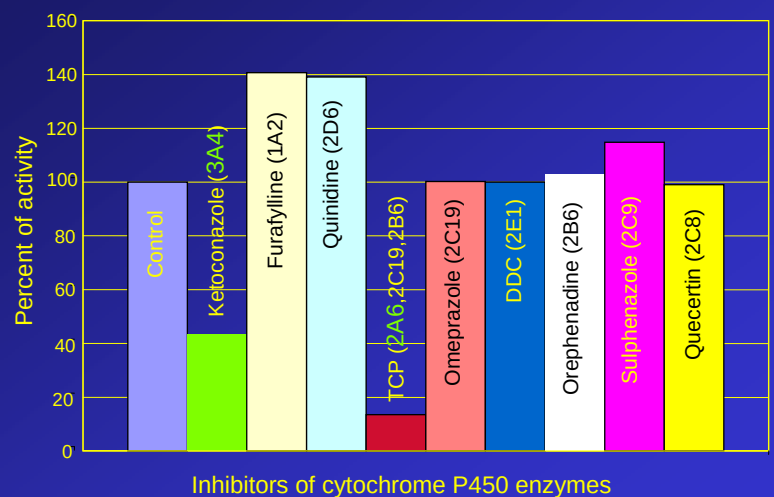
LC-MS Detection of Metabolites of Formononetin, Prunetin and Biochanin A



Phase I Metabolism of Biochanin A, Prunetin and Formonetin to Genistein and Daidzein

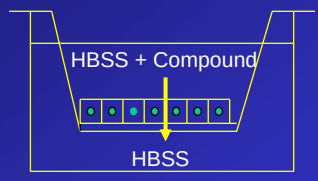


Inhibition of O-Demethylation of Formononetin by Selective Inhibitors of Cytochrome P450

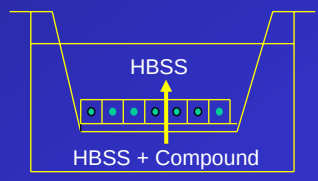


Caco-2 Cell Monolayer System to Study Absorption Across Human Intestinal Mucosa

- Uptake of compounds across intestinal mucosa is determined by a combination of processes including Paracellular diffusion, Transcellular diffusion, Facilitated transport, and Metabolism.
- Facilitated transport (i.e.,P-glycoprotein) can be probed using specific inhibitors and measuring transport rates in opposite directions
- Use of mass spectrometry enhances the amount of information available concerning substrate metabolism and allows multiple compounds to be studied simultaneously.



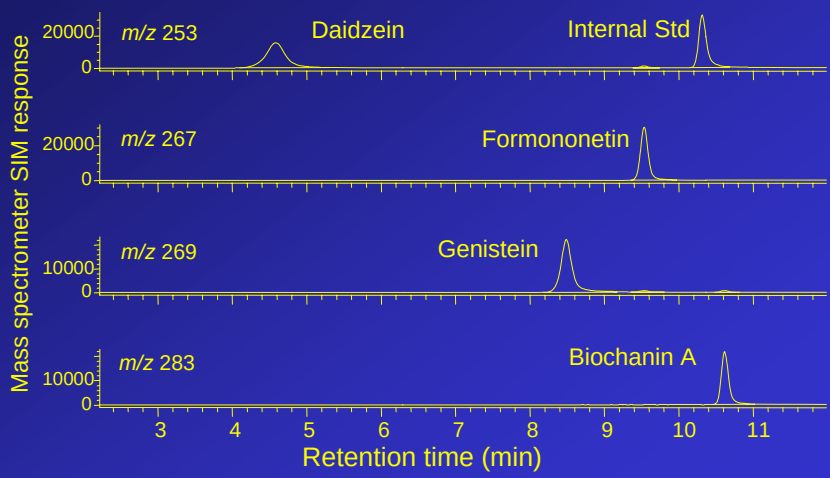
Apical to Basolateral



Basolateral to Apical

LC-MS Analysis of Red Clover Isoflavones in the Caco-2 Culture Medium

Reversed Phase HPLC and Negative Ion APCI



Apparent Permeability Coefficients of Red Clover Isoflavones through the Caco-2 Monolayer

Papp[1] cm/sec ($\times 10^{-5}$)	Daidzein 50 μM	Genistein 50 μM	Formononetin 50 μM	Biochanin A 50 μM
AP to BL[2]	3.10 ± 0.48	4.06 ± 0.65	4.62 ± 0.23	5.47 ± 0.23
BL to AP[3]	2.76 ± 0.19	4.56 ± 0.12	4.29 ± 0.13	5.11 ± 0.25

[1] P_{app} = Apparent Permeability Coefficients, cm/sec ($\times 10^{-5}$)
[2] AP→BL = Apical to Basolateral transport
[3] BL →AP = Basolateral to Apical transport
Data are expressed as mean ± SD, n = 3

Caco-2 Monolayer Papp Coefficients of Red Clover Isoflavones from a Capsule Extract

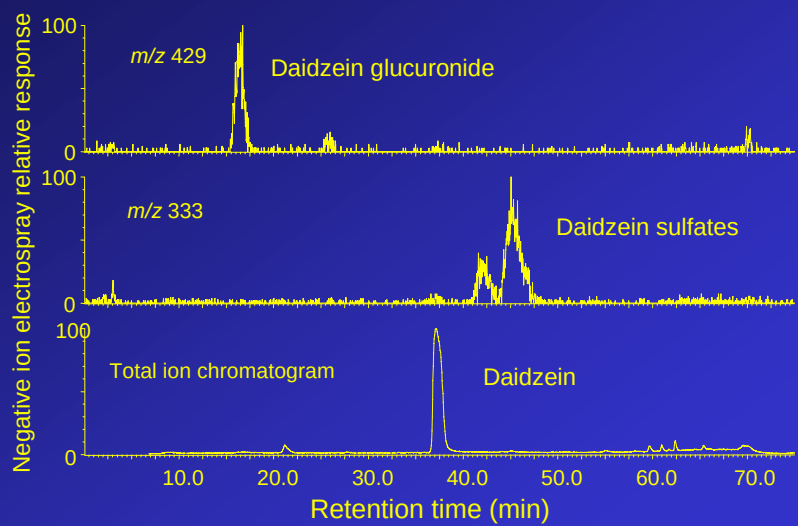
Papp[1] cm/sec ($\times 10^{-5}$)	Daidzein	Genistein	Formononetin	Biochanin A
AP to BL[2]	4.36 ± 0.07	4.36 ± 0.23	5.78 ± 0.23	4.95 ± 0.24
BL to AP[3]	4.22 ± 0.36	5.05 ± 0.33	5.50 ± 0.24	5.15 ± 0.25

[1] P_{app} = Apparent Permeability Coefficients, cm/sec ($\times 10^{-5}$)
[2] AP→BL = Apical to Basolateral transport
[3] BL →AP = Basolateral to Apical transport
Data are expressed as mean ± SD, n = 3

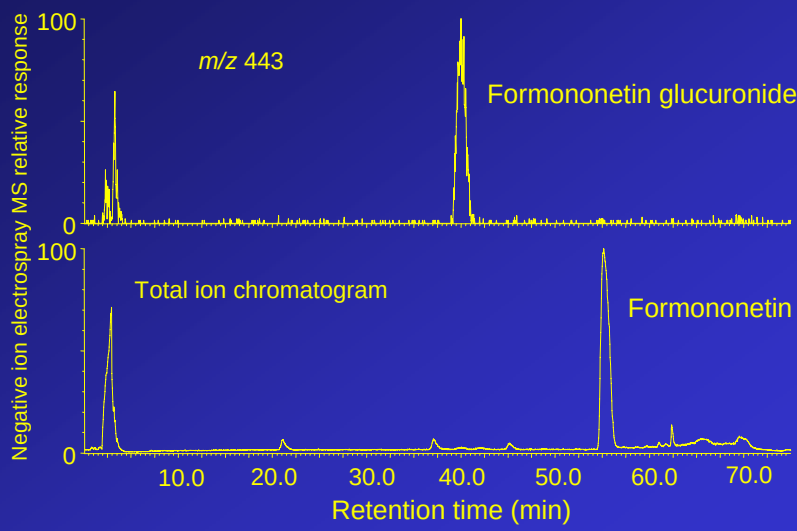
Caco-2 Cell Monolayer System to Study Metabolism of Red Clover Isoflavones

- The metabolism of genistein, daidzein, biochanin A, and formononetin by the intestinal mucosa was investigated by incubating each isoflavone at 50 μM with a Caco-2 cell monolayer for 4 hr at 37 ºC.
- Metabolites were identified using LC-MS and LC-MS-MS.
- Unlike hepatic metabolism involving cytochrome P450 enzymes, no O-demethylation or other Phase I metabolites were detected.
- Phase II glucuronides and sulfates were observed.

LC-MS Analysis of Daidzein Metabolites after Incubation with Caco-2 Cells



LC-MS Analysis of Formononetin Metabolites after Incubation with Caco-2 Cells



Clinical Assessment of Red Clover

Phase 1 Clinical Design

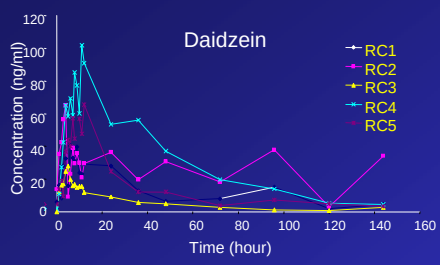
- 15 women were recruited and placed on dietary restrictions for 3 weeks.
- Subjects were randomized into 3 groups and administered a single oral dose of red clover capsules containing 40 mg, 80 mg or 120 mg isoflavones.
- Women were monitored hourly in the GCRC for the first 24 h. Side effects were monitored for 1 week.
- Blood samples were drawn at baseline, hourly for the first 12 h, then at 24, 36, 48, 72, 96, 120, and 144 h.
- Urine was collected for the first 24 h.
- Estrogenic isoflavones in serum and urine were measured using LC-MS

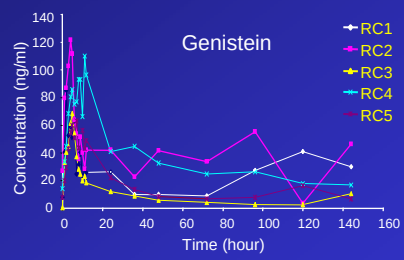
Phase 1 Toxicity Results for the Red Clover Extract

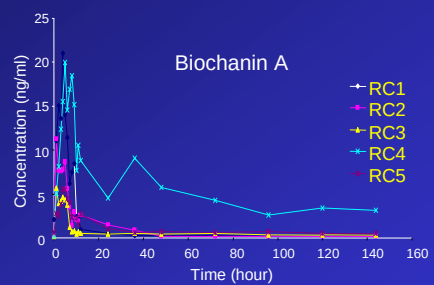
No acute indications of toxicity were detected

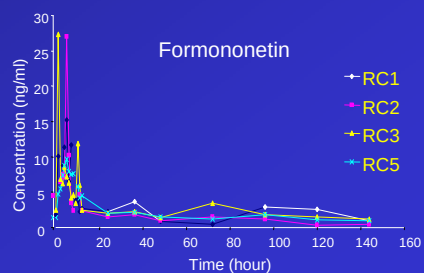
- No nausea or emesis
- No acute discomfort
- No changes in blood pressure or heart rate
- No clinical chemistry abnormalities

LC-MS Time-concentration Curves of Isoflavones in Serum of Women Receiving 40 mg Red Clover Extract

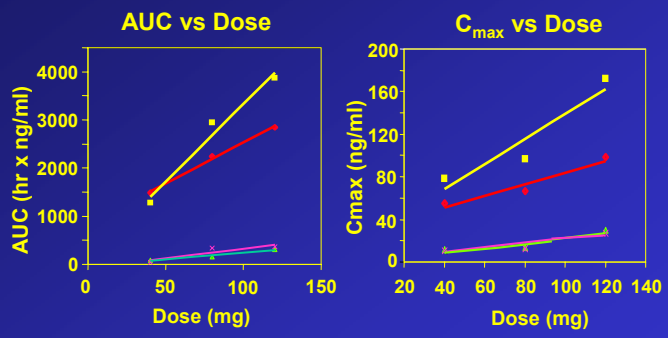








Linear Regression Analysis of AUC and C_{max} vs. Dose For Daidzein, Genistein, Biochanin A and Formononetin

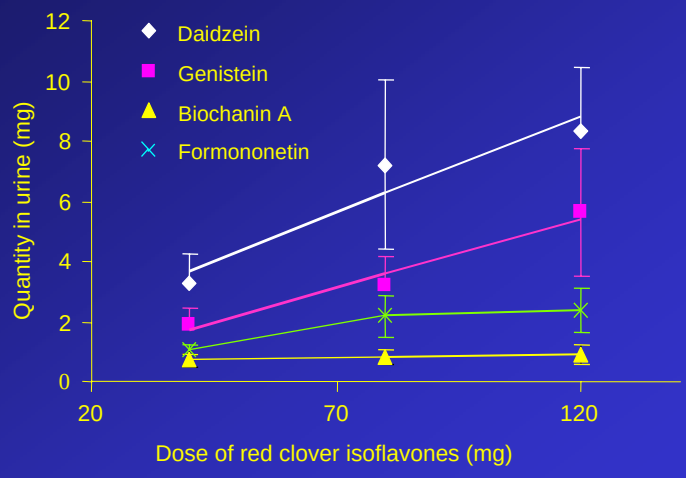


R^2 = 0.9950 Daidzein, 0.9737 Genistein
0.9557 Biochanin A, 0.8096 Formononetin

R^2 = 0.8907 Daidzein, 0.9325 Genistein
0.8012 Biochanin A, 0.8660 Formononetin

Key: ♦ Daidzein ■ Genistein ▲ Biochanin A × Formononetin

24-Hour Urinary Excretion of Daidzein, Genistein, Biochanin A and Formononetin



Clinical Assessment

Phase 2 Clinical Trial of Safety and Efficacy
Randomized, Double-blind, Placebo-controlled

- 88 peri-menopausal women
- 4 arms (22 women per arm)
  - **Black cohosh**
  - Red clover
  - Prempro
  - Placebo
- Daily dosing for 1 year
- Primary endpoint: reduction of hot flashes

Phase 2 Clinical Trial – to date

- > 500 women have been screened
- 60/88 women currently enrolled
- Only 1 withdrawal (subject moved out of area)
- No serious side effects reported
- All biochemical parameters, e.g., liver enzymes, have been normal

Conclusions

Metabolism of Red Clover Isoflavones

- The abundant isoflavone formononetin is O-demethylated by CYP3A4 and CYP2A6 to form the more estrogenic daidzein.
- The abundant red clover isoflavone biochanin A and less abundant prunetin are O-demethylated by CYP3A4 and CYP2A6 to form the more estrogenic genistein.
- Red clover isoflavones can be conjugated in the intestine and in the liver to form monoglucuronides or monosulfates which are excreted in the urine and possibly in the bile.

Conclusions

Pharmacokinetics of Red Clover Isoflavones

- AUC and C_{max} increased linearly with dose
- $T_{1/2}$ of genistein and daidzein >12 h and much longer than formononetin and biochanin A
- Serum concentrations of genistein and daidzein >> biochanin A and formononetin due to metabolic conversion instead of lower intestinal absorption
- Urinary recovery of genistein and daidzein >100% due to metabolic conversion of formononetin and biochanin A to genistein and daidzein

Phase II study of red clover and black cohosh vs. Prempro and placebo is in progress.

Conclusions

Standardization of Botanical Dietary Supplements

- Material should be botanically authenticated
- Active constituents should be identified
- Composition should be chemically standardized
- Activity should be biologically standardized
- Should be tested for contamination by pesticides, herbicides, microbes, and heavy metals
- Should investigate PK and metabolism of active constituents
- Should test for botanical-drug interactions
- Clinical trials of safety and efficacy needed

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