

## Evaluation of Ex Vivo Human Skin Permeation of Genistein and Daidzein

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**The percutaneous absorption of genistein (GEN) and daidzein (DAI), whose oestrogenic-like activity is well known, is scantily investigated. In this work the ability of GEN and DAI to reach therapeutic steady-state plasma concentrations following transdermal administration was studied. The skin permeation studies were conducted by using modified Franz diffusion cell and human epidermis as a membrane. PEG400 was the most effective vehicle for both molecules. On the basis of the ex vivo permeation results and estimating therapeutic plasma concentration, we assume that pure GEN can be efficaciously administered by the transdermal route.**

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**Keywords** Genistein, Skin Permeation, Soy Extract

Phytoestrogens are plant-derived nonsteroidal compounds that possess estrogen-like biological activity. Among all classes of phytoestrogens that have been reported, lignans, coumenstans, and isoflavones appear of particular interest from a health perspective and in particular genistein (GEN) and daidzein (DAI). These naturally occurring isoflavones derived from soybeans have shown estrogenic activity. Epidemiological studies suggest that an elevated intake of DAI and GEN reduced the incidence of many hormone-dependent diseases (Glazier and Bowman 2001). Researchers demonstrated that such isoflavones reduce the risk of breast and prostate cancer (Lee et al. 1991; Fritz et al. 1998; Jacobsen, Knutsen, and Fraer 1998), as well as cardiovascular diseases (Anderson, Johnstone, and Cook-Newell 1995), and improve bone health in an animal model (Arjmandi et al. 1996). Moreover, they have been proposed as an alternative to conventional hormone replacement therapy for the treatment of postmenopausal diseases (Albertazzi and Purdie 2002; Squadrito et al. 2003).

Interest in phytoestrogens and in particular DAI and GEN is mainly because they show a higher binding affinity for human estrogen receptor  $\beta$  (hER $\beta$ ) than receptor hER $\alpha$ . The two receptors differ in their tissue distribution, and hER $\beta$  is distributed in the cardiovascular system, urogenital tract, bone tissue, and central

nervous system (Bovee et al. 2004; Kuiper et al. 1997). Moreover, hER $\beta$  way have a fundamental role against hyperproliferation and carcinogenesis in breast, prostate, and gastrointestinal tract (Bovee et al. 2004). Nevertheless, the recent studies and reviews show contradictory evidences as to the benefits of phytoestrogens (Arjmandi & Smith 2002; Kreijkamp-Kasper et al. 2004).

Among food, soybeans contain the highest amount of isoflavones that are present in four chemical forms: the aglycones (GEN and DAI), malonyl glucosides, acetylglucosides, and the glycosides (genistin and daidzin) which are the most abundant in the raw material. Several studies on soybeans and different soy-protein products have shown high variability in concentration and composition. The relative ratio between the conjugated and unconjugated forms depends on the type and extent of processing soybeans. Moreover, genistin and daidzin are readily hydrolyzed by intestinal bacteria into the corresponding aglycones (Zubik and Meydani 2003); as a consequence the apparent bioavailability of GEN and DAI is not different after oral administration of the glycosides or the aglycones (Zubik & Meydani 2003). The latter forms were found to be the active chemical forms for both the receptors of estradiol (Bovee et al. 2004).

In several studies, the pharmacokinetics of orally administered GEN and DAI were compared (Bloedon et al. 2002; Faughnan et al. 2004; Setchell et al. 2001; Setchell et al. 2003a, 2003b; Zhang et al. 1999; Zubik & Meydani 2003). Some discrepancies were observed in the relative bioavailability that in any case was higher for GEN than DAI (Setchell et al. 2001). The gastrointestinal absorption was not linear with the dose and suggests a saturable mechanism of absorption (Setchell et al. 2001; Setchell et al. 2003a), the half-life was found higher for DAI than GEN and ranged from 5 to 10 h (Setchell et al. 2001; Setchell et al. 2003a, 2003b). On the basis of pharmacokinetic characteristics, the optimum steady-state plasma concentration of the two isoflavones would be expected after frequent and modest intakes of GEN and DAI during all day rather than a single high dose (Setchell et al. 2003a).

Based upon these considerations, transdermal administration can be a suitable route of administration of GEN and DAI because it can assure prolonged release of low dose of active

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principles. Nevertheless, GEN and DAI transdermal absorption was scantily investigated. Vanttnen and Moravcova (2001) evaluated the ability of the soy isoflavones to penetrate the skin structure by monitoring plasma and urine levels. The pure isoflavones were applied to the skin as a suspension in extra virgin olive oil (10 mg GEN or DAI in 1 g oil). In this study both the capacity of GEN and DAI to permeate the skin barrier and to reach the systemic circulation was implicitly assumed. Plasma and urinary recoveries might indicate an accumulation of the isoflavones in the skin structures followed by a slow desorption. Moreover, the applied dose was reached, but not maintained, at levels useful for therapeutic purposes (Vanttnen and Moravcova 2001).

The present work is an *ex vivo* preliminary evaluation of the ability of these isoflavones to reach therapeutic steady-state plasma concentrations following transdermal administration. The effect of the other components of a dry extract on the GEN and DAI skin permeation also was investigated by comparing their skin permeation to that of pure GEN and DAI.

To assay the influence of the vehicle on the isoflavones skin permeation, experiments used saturated solutions of dry soy extract in water, oleic acid, Labrasol<sup>®</sup>, Transcutol<sup>®</sup>, polyethylene glycol 400, and propylene glycol were performed. The skin permeation studies conducted by using the pure isoflavones were performed in the vehicle in which pure DAI and GEN showed the highest solubility. The skin permeation studies used modified Franz diffusion cell and the human stratum corneum and epidermis as a membrane.

## MATERIALS AND METHODS

Genistein (GEN) and daidzein (DAI) (Figure 1) were supplied by Sigma-Aldrich (USA). Soybean dry extract (Genimax<sup>®</sup>) was purchased from Sochim International (Italy); Oleic acid (OA) was supplied by Esperis (Italy); polyethylene glycol 400 (PEG400) and propylene glycol (PG) came from ACEF (Italy); diethylene glycol monoethyl ether, Transcutol<sup>®</sup> (TR), and caprylocaproyl macrogol-8 glycerides, Labrasol<sup>®</sup> (LB) was supplied by Gattefossè (France).

All the solvents were of analytic grade, unless specified.

## Solubility

The solubility of the dry soy extract in water, OA, PEG400, PG, TR, and LB was obtained by equilibrating a large excess of the extract and vehicle for at least 48 hr. Each solution was stirred vigorously throughout the experiment and the temperature was maintained at  $32 \pm 1^\circ\text{C}$ . After equilibration, an aliquot of sample was filtered quickly with a membrane filter (Millipore Millex-HV, pore size  $0.45 \mu\text{m}$ , Millipore, USA). Then the solution was appropriately diluted with methanol, and the concentration of GEN and DAI was determined by HPLC, according to the method described below. The solubility of GEN and DAI as pure isoflavones also was determined in the vehicle showing the highest solubilized amount by using the extract.

## Ex Vivo Skin Permeation Study

Human skin was obtained from abdomen with surgical operation from a single donor and stratum corneum and epidermis (SCE) was prepared as described in a previous work (Minghetti et al. 1999a). Each SCE sample was carefully mounted on a modified Franz-type diffusion cell with the dermal side in contact with the receptor phase, made of a mixture 0.9% NaCl solution/TR (80/20, v/v). At the beginning of the experiment, 0.5 ml of a saturated solution with an excess of dry extract in each of the selected vehicles was applied to the diffusion cell as donor phase. The permeability of pure GEN and DAI by using the vehicle in which the extract showed the highest phytoestrogen solubilized amount also was performed. After 1, 3, 5, 7 and 24 hr, 0.2 ml samples were withdrawn from the receiver compartment. Sink conditions were always maintained. The GEN and DAI were assayed by the method described below.

To evaluate the amount of DAI and GEN retained in the SCE after 24 hr, the saturated solution was removed and the surface of the SCE was washed with methanol, cut into small pieces, and added to methanol. The suspension, soaked for 30 min, was stored for 12 hr and then filtered. The concentrations of GEN and DAI in the collected samples were determined by the isocratic method described in the next section.

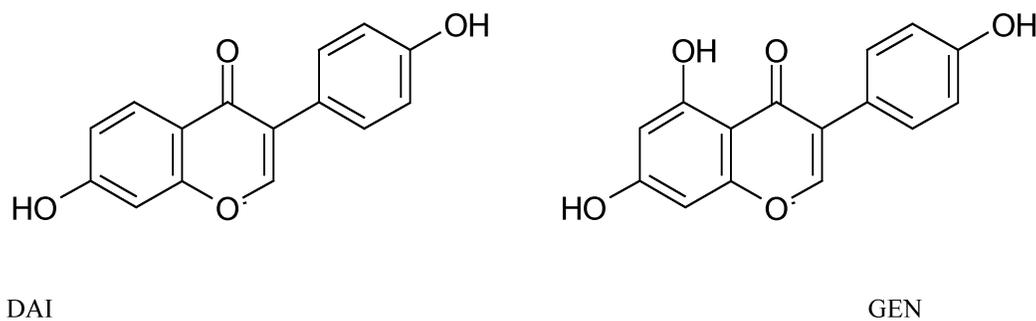


FIG. 1. Chemical structures of DAI and GEN.

### GEN and DAI Assay

The analytical instrument was an HPLC (HP 1100, Chemstations, Hewlett Packard, USA). Conditions of the isocratic method are injection volume at 10  $\mu$ l, at a flow rate of 1 ml/min, and ultraviolet absorbance was monitored at 260 nm. Separation of the individual isoflavones was obtained on a C18 reverse-phase column (Hypersil, 5  $\mu$  m Spherisorb ODS2, 4.6  $\times$  200 mm, Waters, UK). The mobile phase was methanol-2 mmol/L and ammonium acetate (55/45, v/v) and temperature was fixed at 25°C. A standard calibration curve (0.1–50  $\mu$ g/ml) for each molecule was used. The detection limit was 0.03  $\mu$ g/ml for DAI and 0.05  $\mu$ g/ml for GEN. Resolution from the background noise was adequate at this level. The other substances of the soy extract did not interfere in DAI and GEN determination. The retention time of GEN and DAI was 10 min and 7 min, respectively.

Conditions of the gradient method were injection volume at 10  $\mu$ l, at a flow rate of 1 ml/min, and ultraviolet absorbance was monitored at 260 nm. Separation of the individual isoflavones was obtained on a C18 reverse-phase column (Hypersil). Temperature was fixed at 25°C. The mobile phase was 100% of 10 mmol/L ammonium acetate (0.1% trifluoroacetic acid) held isocratic for the first 2 min and then decreased to 50% at a constant gradient from 2 to 24 min, then finally held isocratic with 50% acetonitrile and 50% 10 mmol/L ammonium acetate (0.1% trifluoroacetic acid) for 5 min, before being returned to the original composition of 100% 10 mmol/L ammonium acetate (0.1% trifluoroacetic acid). The retention time of GEN and DAI was 25 min and 22 min, respectively.

### Data Analysis

The cumulative amount permeated through the SCE per unit of area was calculated from the concentration of each substance in the receiving medium and plotted as a function of time. Each data point on the plot represents a mean of triplicate permeation experiments. The flux (J) was determined as the slope of the linear portion of the plot. The permeability coefficient was calculated as:

$$K_p = J_{\max}/S \quad [1]$$

where  $K_p$  is the permeability coefficient (cm/h),  $J_{\max}$  is the maximum flux ( $\mu$ g/cm<sup>2</sup>/hr), and S is the drug donor concentration corresponding to the drug solubility in the vehicle ( $\mu$ g/mL).

The predicted plasma-steady state concentration was obtained using the following equation:

$$C_{ss} = J_{\max} \text{ TTS area}/Cl \quad [2]$$

where  $C_{ss}$  is the plasma steady-state concentration ( $\mu$ g/L), TTS area is the hypothetical area of a transdermal therapeutic system (cm<sup>2</sup>), and Cl is the systemic clearance (L/hr) (Minghetti et al. 1999b).

TABLE 1

Solubility of GEN and DAI contained in the dry soy extract

Vehicle	GEN (mg/ml $\pm$ s.d.)	DAI (mg/ml $\pm$ s.d.)
Polyethylene glycol 400	8.51 $\pm$ 0.43	8.53 $\pm$ 0.46
Labrasol®	8.05 $\pm$ 0.21	5.73 $\pm$ 0.12
Transcutol®	6.83 $\pm$ 0.03	6.86 $\pm$ 0.03
Propylene glycol	4.34 $\pm$ 0.03	2.61 $\pm$ 0.02
Oleic acid	0.025 $\pm$ 0.008	0.001 $\pm$ 0.001
Water	0.015 $\pm$ 0.005	0.013 $\pm$ 0.01

Virtual log P was obtained within the modeling software VEGA (Pedretti, Villa, and Vistoli 2002) using a method reported by Gaillard et al. (1994). Test-t for significant differences between means was performed by program SPSS 1 (Spss Inc., USA). Differences were considered significant at the  $p < 0.05$  level.

### RESULTS AND DISCUSSION

The dry soy extract, analyzed by the gradient method, exclusively contained the aglycones GEN and DAI in the amount of 24% m/m, corresponding to 11.7% m/m GEN and 12.3% m/m DAI and indicating that the extraction method caused the hydrolysis of the conjugated forms of the isoflavones. The solubilities of GEN and DAI contained in the dry extract in the selected vehicles are reported in Table 1. The solubility of both the isoflavones in water was the lowest and very close to that measured in OA (less than 0.1 mg/ml, Table 1). GEN was more soluble than DAI in PG and LB.

The main permeation parameters of GEN and DAI contained in the dry extract are reported in Table 2. The absence of skin permeability observed when water or OA was used was attributed to the very low solubility of both isoflavones in these vehicles that probably did not permit permeation of a quantifiable amount of both the molecules in the receiver medium. The lack of enhancement effect of LB and TR can be attributed to the fact that they act more as a solubilizing agent of substances in the skin than a perturbing agent of the barrier properties of the skin itself (Minghetti et al. 2001). Taking into account these results and considering that GEN and DAI have in all the saturated solutions the same thermodynamic activity, PEG400 could be considered the most effective vehicle for both molecules.

The solubilities of the pure GEN and DAI in PEG400 were comparable (Table 3) and about one-third higher than those determined from the extract. The results on the skin permeation of pure isoflavones in PEG400 are reported in Table 4. The highest retained amount of DAI with respect to GEN can be due to higher log P that favors the partition of the molecules in the lipidic network of the stratum corneum. The permeated amount of pure GEN after 24 hr was ~4-fold higher than that of pure DAI. This feature was in agreement with the results of in vivo

TABLE 2  
Permeation parameters of GEN and DAI from the dry soy extract saturated vehicles

Vehicle	GEN			DAI		
	Permeated amount after 24 hr ( $\mu\text{g}/\text{cm}^2 \pm \text{s.d.}$ )	Flux ( $\mu\text{g}/\text{cm}^2/\text{hr}$ )	Permeability coefficient ( $K_p$ ) ( $\text{cm}^2/\text{hr}$ )	Permeated amount after 24 hr ( $\mu\text{g}/\text{cm}^2 \pm \text{s.d.}$ )	Flux ( $\mu\text{g}/\text{cm}^2/\text{hr}$ )	Permeability coefficient ( $K_p$ ) ( $\text{cm}^2/\text{hr}$ )
Polyethylene glycol 400	31.7 $\pm$ 8.7	1.3 $\pm$ 0.4	$1.5 \times 10^{-4}$	23.9 $\pm$ 11.9	1.0 $\pm$ 0.5	$1.2 \times 10^{-4}$
Labrasol	5.6 $\pm$ 0.4	—**	—	—*	—	—
Transcutol	5.8 $\pm$ 0.2	—**	—	—*	—	—
Propylene glycol	12.3 $\pm$ 3.2	0.5 $\pm$ 0.1	$1.2 \times 10^{-5}$	9.8 $\pm$ 3.9	0.4 $\pm$ 0.2	$1.5 \times 10^{-5}$
Oleic acid	—*	—	—	—*	—	—
Water	—*	—	—	—*	—	—

\*Not detectable, \*\*lower than 0.1  $\mu\text{g}/\text{cm}^2/\text{hr}$ .

study that reported higher plasma level of GEN after transdermal administration (Vantnen and Moravcova 2001).

The permeated amounts of pure GEN and DAI were 4-fold and 2-fold higher, respectively, than those obtained by using the dry soy extract. The higher fluxes of pure GEN cannot be attributed exclusively to the higher solubility in PEG400. Indeed, the  $K_p$  of the pure isoflavones resulted in about 4-fold higher than that determined for isoflavones contained in the dry soy extract (Table 2 and Table 4). Thus, the reduction of the fluxes can be attributed mainly to the effect of other substances contained in the dry soy extract that can inhibit and/or compete with the GEN partition in the stratum corneum.

As therapeutical plasma concentrations ( $C_t$ ) of DAI and GEN were not available in the literature, they were estimated considering the therapeutic concentration of estradiol after transdermal

administration ( $C_t = 35 \text{ pg/mL}$  [Scarabin et al. 1997]) and the relative potency on hER $\beta$  (REP) of the two isoflavones with the steroidal hormone (REP DAI:  $1.1 \times 10^{-4}$ ; REP GEN:  $1.1 \times 10^{-2}$  [Bovee et al. 2004]). The estimated  $C_t$  of DAI and GEN were 3.9 mg/L and 39  $\mu\text{g}/\text{L}$ , respectively.

As reported in the Data Analysis section (eq. 2), the ex vivo SCE permeation data allowed us to estimate the rate of input of a substance into the systemic circulation. It should be possible to evaluate the achievable plasma levels combining the ex vivo permeability data with the systemic clearance of DAI and GEN as reported in literature—30.09 L/hr and 21.85 L/hr, respectively (Setchel et al. 2003b). Assuming for a transdermal delivery system an area comparable to that of the largest transdermal patch available on the market (40  $\text{cm}^2$ ), the estimated plasma concentrations ( $C_{ss}$ ) from the ex vivo experiments conducted by using the pure isoflavones resulted in  $C_{ss} = 13.0 \mu\text{g}/\text{L}$  for GEN and  $C_{ss} = 2.2 \mu\text{g}/\text{L}$  for DAI. The  $C_t/C_{ss}$  ratio for DAI was 1772 and was too high to consider transdermal administration. In the case of GEN, the  $C_t/C_{ss}$  ratio was equal to 3; therefore transdermal administration could be taken into consideration. Indeed, isosorbide dinitrate, which presented a  $C_t/C_{ss}$  ratio of 10.23 (Minghetti 1999b), is available on the market as a patch.

TABLE 3  
Relevant physicochemical parameters of GEN and DAI

	Molecular weight	Melting point ( $^{\circ}\text{C}$ )	Virtual log P*	Solubility** (mg/ml $\pm$ s.d.)
DAI	270.24	320	3.16	11.6 $\pm$ 0.1
GEN	254.24	303	2.87	12.3 $\pm$ 0.7

\*Calculated by VEGA software; \*\*determined in PEG 400.

TABLE 4  
Permeation parameters of pure GEN and DAI from PEG400 (mean  $\pm$  s.d.)

	Retained amount after 24 hr ( $\mu\text{g}/\text{cm}^2$ )	Permeated amount after 24 hr ( $\mu\text{g}/\text{cm}^2$ )	Flux ( $\mu\text{g}/\text{cm}^2/\text{hr}$ )	Permeability coefficient ( $K_p$ ) ( $\text{cm}^2/\text{hr}$ )
DAI	80.7 $\pm$ 3.9	37.3 $\pm$ 16.8	1.7 $\pm$ 0.8	$1.5 \times 10^{-4}$
GEN	36.0 $\pm$ 0.4	155.7 $\pm$ 18.2	7.1 $\pm$ 0.8	$5.8 \times 10^{-4}$

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