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Liquid chromatography coupled with multi-channel electrochemical detection for the determination of daidzin in rat blood sampled by an automated blood sampling system

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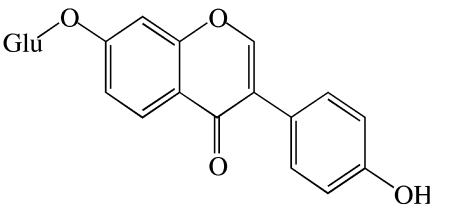
Abstract

Daidzin, a soy-derived biologically active natural product, has been reported to inhibit mitochondrial aldehyde dehydrogenase and suppress ethanol intake. This paper describes a method for the determination of daidzin in rat blood. After administration of daidzin, blood samples were periodically collected from awake, freely moving animals by a Culex automated blood sampler. Daidzin was extracted from 50 μl of diluted blood (blood and saline at a ratio of 1:1) with ethyl acetate. Chromatographic separation was achieved within 12 min using a microbore C_{18} (100×1.0 mm) 3 μm column with a mobile phase containing 20 m*M* sodium acetate, 0.25 m*M* EDTA, pH 4.3, 4% methanol and 11% acetonitrile at a flow-rate of 90 μl/min. Detection was attained using a four-channel electrochemical detector with glassy carbon electrodes using oxidation potentials of +1100, 950, 850, 750 mV vs. Ag/AgCl. The limit of detection for daidzin in rat plasma was 5 ng/ml at a signal-to-noise ratio of 3:1. The extraction recovery of daidzin from rat plasma was over 74%. Linearity was obtained for the range of 25–1000 ng/ml. The intra- and inter-assay precisions were in the ranges of 2.7–6.6 and 1.9–3.7%, respectively. This method is suitable to routine in vivo monitoring of daidzin in rat plasma.

Keywords: Daidzin

1. Introduction

Daidzin, a glucosylated isoflavone (glycosylated daidzein), is found to be abundant in soybeans [1] as well as the root of *Puerariae lobata*, a traditional Chinese medicinal plant [2]. Fig. 1 shows the structure of daidzin. As one of the major isoflavone



Daidzin (DIN)

Fig. 1. Structure of daidzin.

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compounds, daidzin has been implicated to have various biological activities, such as antioxidant, estrogenic, antiestrogenic and anticarcinogenic effects [3–8]. Mechanistic studies have indicated that daidzin inhibits cyclic AMP phophosdiesterase [9] and induces differentiation in murine erythroleukemia cells [10]. In addition, daidzin has been found to have a very unique property of decreasing voluntary alcohol consumption in different animal models [11–13]. It has been shown to be a very potent and selective inhibitor of human mitochondrial aldehyde dehydrogenase, which could explain its role in inhibiting voluntary alcohol consumption [14]. Metabolites of daidzin administered in rats have been identified [15,16].

Liquid chromatography (LC) coupled with electrochemical detection (ED) has been demonstrated as a sensitive and selective method for detecting daidzin and related isoflavones mostly in food [17–20]. In most studies, this glycosylated isoflavone is subjected to enzymatic hydrolysis and measured as daidzein [21–23]. Reports on the determination of intact daidzin in biological fluids are few [24].

This study describes a simple, sensitive and selective LC–ED method for the determination of daidzin in rat plasma following intravenous (i.v.) administration. By applying an electrode potential, oxidation reactions of daidzin will take place, which give rise to the detector signal. Samples were periodically collected by an automated blood sampling device, which has been proven to be a powerful tool for unattended pharmacokinetic and pharmacodynamic studies involving low stress freely moving rats [25–27].

2. Experimental

2.1. Apparatus

The LC–ED system (all from BAS, West Lafayette, IN, USA) was comprised of a chromatographic pump (PM-92e), a guard column (C_{18}, 3 μm, 14× 1.0 mm), a microbore analytical column (C_{18}, 3 μm, 100×1.0 mm), and a multi-channel amperometric detector (Epsilon) coupled to a quad-glassy carbon working electrode. Applied potential were +750, 800, 850 and 950 mV vs. Ag/AgCl. Samples were injected by using a CMA/200 refrigerated autosampler (CMA/Microdialysis, Stockholm, Sweden) with a 10 μl loop. Data were acquired and integrated using BAS ChromGraph version 2.0.01 chromatography software.

The blood collecting system consisted of a freely moving rat containment device (Raturn, BAS) [28], and an automated blood sampler (Culex, BAS). The blood samples were collected into a refrigerated fraction collector (HoneyComb, BAS) maintained at 4 °C.

2.2. Chemicals and reagents

Daidzin was purchased from Indofine Chemical (Somerville, NJ, USA). Acetonitrile, methanol and ethyl acetate were of HPLC grade (Burdick and Jackon, Muskegon, MI, USA). Reagent grade water was prepared from laboratory-deionized water using a NANOpure system (Barnstead/Thermolyne, Dubuque, IA, USA). Dimethyl sulfoxide (DMSO) was analytical-reagent grade purchased from Fisher Scientific (Fair Lawn, NJ, USA). Sodium acetate and ethylenedinitrilotetraacetic acid disodium salt (EDTA) were of analytical-reagent grade (Mallinckordt, Paris, KY, USA).

2.3. Standard curve and quality control samples

Daidzin was dissolved in methanol at a concentration of 1 mg/ml using a brown vial and stored at a −20 °C until use. This stock was diluted, as needed, in saline to a concentration of 10 μg/ml and spiked into pooled rat plasma to yield final concentrations of 25, 50, 100, 200, 500, 1000 ng/ml. These spiked samples were used to construct the standard curve. Quality control (QC) samples were prepared in pooled rat plasma to contain concentrations of daidzin within the standard curve range.

2.4. Sample preparation

A total of 150 μl of blood solution, which contained 75 μl of rat blood and 75 μl of saline containing heparin, comprised each sample. The solution was centrifuged at 2000 *g* for 10 min. Then two aliquots of plasma, 50 μl each, were transferred to 1.7 ml Eppendorf tubes, one for immediate

analysis, the other for storage at −20 °C as a backup. Ethyl acetate (0.8 ml) was added to the plasma, vortex-mixed for 2 min, and centrifuged at 5600 *g* for 6 min. Following centrifugation, 700 μl of the clear supernatant was transferred to another centrifuge tube, dried under nitrogen and reconstituted with 20 μl of mobile phase. A volume of 10 μl of the solution was injected by autosampler.

2.5. Assay validation

2.5.1. Calibration

A calibration curve for daidzein was constructed by plotting peak height at +850 mV vs. concentration (ng/ml). The weighted (*x*) linear regression was fitted over the concentration range of 25–1000 ng/ml.

2.5.2. Accuracy and precision

The inter- and intra-assay validation was performed by assaying QC samples (50, 200, and 1000 ng/ml) in triplicate on three different days. The accuracy and precision were reported as the bias (%) and the RSD (%), respectively.

2.5.3. Recovery

Daidzin solution, 0.4 mg/ml in saline, was spiked into rat plasma and mobile phase, respectively, to yield concentrations of 300 and 2000 ng/ml. The spiked rat plasma samples were extracted and reconstituted according to the procedures described in Section 2.4. The 2000 ng/ml samples were further diluted five times with mobile phase to make sure that they fell within the range of the calibration curve. The 300 ng/ml samples were assayed directly. The recovery rate was calculated by comparing the daidzin peak areas of the spiked and extracted rat plasma with the spiked mobile phase at the corresponding concentrations, factoring the enrichment coefficient.

2.5.4. Stability

Daidzin spiked plasma samples were extracted immediately, and being kept in the dark at 4 °C for 24 h. Processed samples were injected or stored at 4 °C for 8 h and then injected.

2.6. Preliminary animal study

Traditional pharmacokinetic studies involve intermittent blood sampling and subsequent determination of drug concentrations in blood or plasma. The Culex automated blood sampler provides a mean for blood withdrawal at preprogrammed intervals, which is more accurate and less labor intensive [25,26,29]. Male Sprague–Dawley rats weighting 280–350 g were implanted with a jugular vein catheter (CX-2010, BAS) and/or femoral vein catheter (CX-2020, BAS). After surgery, the rats were installed in the Raturn, and allowed to recover for 1 day with free access to food and water. The rats were dosed i.v. with daidzin through the femoral vein catheter. A 75 μl blood sample was withdrawn from the jugular vein into a vial, containing an equal volume of heparine/saline and kept in a refrigerated fraction collector according to a preset schedule.

Two different daidzin dosing solutions were used, although, both were administered at the same level of 5 mg/kg of body mass. The first was of standard daidzin in DMSO–saline (20:80) at a concentration of 2 mg/ml. The second solution was a botanical mixture prepared from a nutritional supplement in the following manner: 50 tablets were grounded to a fine powder with a mortar and pestle, 12 g was added to 300 ml methanol–water (80:20) and sonicated for 30 min on ice water. The filtered suspension was dried with a rotary evaporator and 100 mg of dried powder was dissolved in 1 ml of DMSO. A 4-ml volume of saline was added to give a 20 mg/ml solution in 20% DMSO–saline. Quantitation [20] of this solution indicated that 2 ml/kg dose of this solution delivered an equivalent amount of daidzin as the first dose.

3. Results and discussion

3.1. Method development

Based on voltammetric characterization of daidzin, the four-electrode detector was set with applied potentials of +750, 800, 850 and 950 mV. Peak assignment and purity can be carried out with great confidence by comparing the response ratios at different oxidation potentials for standards and sam-

ples [18]. As shown in Fig. 2, daidzin was well separated from any interference under the LC conditions used: 4% methanol, 11% acetonitrile and 85% aqueous buffer (10 m*M* sodium acetate, 0.25 m*M* EDTA, pH 4.3), flow-rate at 90 μl/min.

3.2. Validation of assay

3.2.1. Selectivity

Chromatograms were obtained and compared between the extract of blank rat plasma and spiked rat plasma (Fig. 2). At +850 mV applied potential, no interfering peaks were detected at the retention time of daidzin. Samples could be injected every 12 min.

3.2.2. Linearity

A weighed linear regression of the peak height versus standard concentrations was performed for daidzin. The observed peak heights were linear over the concentration range of 25–1000 ng/ml in rat plasma. The mean values (±SD) ($n=3$) for slope, intercept and r^2 were 155±17, 1543±3455, 0.998±0.002, respectively, for three calibration curve plots.

3.2.3. Limit of detection and quantitation

The detection limit of daidzin in rat plasma was determined to be 5 ng/ml at a signal-to-noise ratio of 3. The lower limit of quantitation was taken as 15 ng/ml.

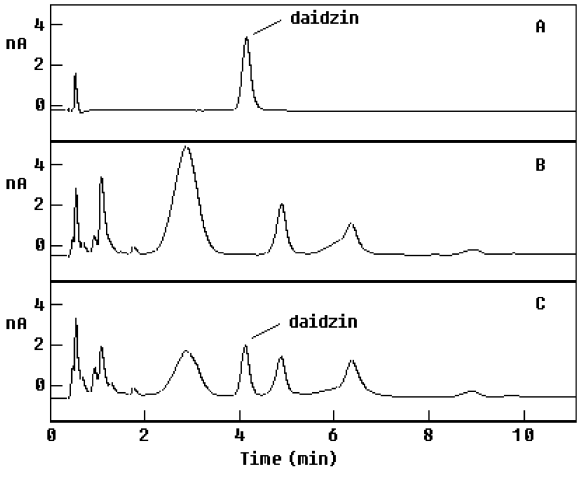

Fig. 2. Chromatograms of daidzin standard (A) blank rat plasma (B) and plasma spiked with 50 ng/ml of daidzin (C). Applied potential: +850 mV vs. Ag/AgCl.

3.2.4. Accuracy and precision

The intra- and inter-day accuracy and precision values for QC samples are presented in Table 1. The precision values (RSD) at the three concentrations in the intra-assay study varied between 2.7 and 6.6% while that of the inter-assay study varied between 1.9 and 3.7%. The accuracy (bias) values for all three concentrations deviated less than 5.6% from the corresponding nominal concentrations.

3.2.5. Extraction recovery

A comparison of neat standard versus plasma-extracted standard indicated that the extraction recovery of the analyte from rat plasma was 74% at concentration of 300 ng/ml and 75% at concentration of 2000 ng/ml. Standard deviations are derived from three independent replicates.

3.2.6. Stability

Daidzin was stable in rat blood kept in the dark and at 4 °C for 24 h. The processed samples experience no significant loss of daidzin when they were stored at 4 °C for 8 h.

3.3. Plasma concentration of daidzin after a single i.v. dosing

The proposed method was used for the determination of daidzin in rat plasma. Fig. 3 illustrates data for a single intravenous dose administration of pure daidzin (filled circles) and daidzin-containing botani-

Table 1
Accuracy and precision of daidzin determination in rat plasma

Added concentration (ng/ml)	Measured concentration (ng/ml)	RSD (%)	Bias (%)
Intra-day ($n=3$)			
50	48.5±2.4	4.9	−3.0
200	209±6	2.7	4.4
1000	958±63	6.6	−4.2
Inter-day ($n=3$)			
50	47.2±1.7	3.7	−5.6
200	211±4	1.9	5.4
1000	980±21	2.1	−2.0

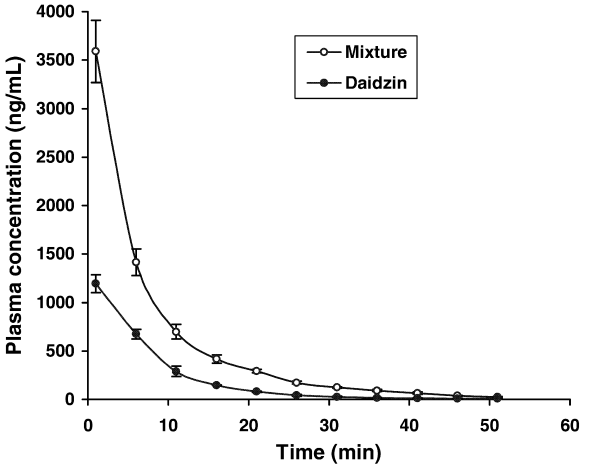

Fig. 3. Mean (±SD) plasma daidzin concentration versus time profile in rats ($n=4$) following a single intravenous administration. Filled circles: administration of pure daidzin at 5 mg/kg. Open circle: administration of daidzin-containing botanical mixture, at a dose equivalent to 5 mg/kg of standard compound.

cal mixture at an equivalent dose (open circles) to four different rats. It is interesting to see that daidzin given as a botanical mixture results in a higher initial plasma concentration as compared to the pure compound. This observation is consistent with the report that crude kudzu extract potentiates the bioavailability of daidzin administered intraperitoneally to hamsters [30]. This impact of the complete botanical mixture on metabolism and elimination rates is not yet clear.

4. Conclusion

An LC–ED method utilizing multi-channel electrochemical detection was developed and evaluated for the determination of daidzin in rat plasma. The automated blood sampling device and the reported method offer several advantages, such as an easy and accurate withdraw of blood, a rapid and clean extraction scheme and a short chromatographic run time.

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