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Short communication

Determination of butein in rat serum by high performance liquid chromatography

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Abstract

A simple high performance liquid chromatography (HPLC) method was developed for the determination of butein in rat serum. The method involved the deproteinization followed by injection into a Luna C8 column. Butein was eluted at 3.8 min at a flow rate of 0.2 ml/min with the mobile phase of acetonitrile–ammonium formate (10 mM, pH 3.0) (35:65, v/v). The standard curve was linear ($r^2 = 0.995$) over the concentration range of 0.1–10 μg/ml. The coefficient of variation (CV) of intra- and inter-assay ranged from 2.7 to 7.5% and 6.0 to 7.5%, respectively. The limit of quantification was 0.1 μg/ml using a serum sample of 50 μl. This method was applied to a pharmacokinetic study after intravenous injection of butein (5 mg/kg) to rats.
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1. Introduction

Butein (3,4,2′,4′-tetrahydroxychalcone) (Fig. 1), a plant polyphenol, possesses the various biological activities, that is, the antioxidant activities [1–3], antinephritic effect [4], the inhibition of epidermal growth factor receptor tyrosine kinase [5,6], the induction of apoptosis in HL-60 and B16 melanoma 4A5 cells [7,8] and antifibrogenic effect in fibrotic rats via the suppression of myofibroblastc differentiation of rat hepatic stellate cells [9]. Butein could be developed as a new therapeutic agent for the prevention and treatment of collagen disposition in chronic liver injury.

It was reported that butein was mainly metabolized to 3-*O*-methylbutein and 3-*O*-methylbutein sulfate in the bile and urine sample obtained from ^{14}C-butein treated rats [10]. There was no method for the determination of butein in biological fluids. It is necessary to develop an analytical method for butein in order to evaluate the pharmacokinetic disposition of butein. The purpose of this paper was to describe and validate a HPLC method using deproteinization for the determination of butein in rat serum. The pharmacokinetics of butein in male Sprague–Dawley rats were also reported.

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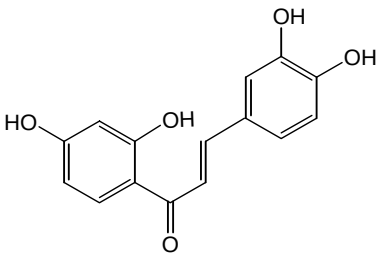

Fig. 1. Chemical structure of butein.

2. Experimental

2.1. Materials

Butein was obtained from Calbiochem–Novabiochem (La Jolla, CA, USA). Methanol and acetonitrile (HPLC grade) were obtained from Burdick & Jackson Inc. (Muskegon, MI, USA) and the other chemicals were of the highest quality available.

2.2. Preparation of calibration standards and quality control samples

Primary stock solutions of butein (1 mg/ml) were prepared in deionized water. Working standard solutions of butein were prepared by diluting each primary solution with deionized water. Rat serum calibration standards of butein (0.1, 0.5, 1.0, 2.0, 5.0, and 10.0 μg/ml) were prepared by spiking appropriate amount of the working standard solutions into drug-free rat serum. Quality control (QC) samples at 0.75, 2.50 and 7.50 μg/ml were prepared in bulk by adding the appropriate working standard solution to drug-free rat serum. The QC samples were aliquoted (50 μl) into polypropylene tubes and stored −20 °C until analysis.

2.3. Sample preparation

Fifty microliter of 10% acetic acid and 100 μl of acetonitrile were added to a 50 μl aliquot of blank serum, calibration standards and QC samples. After vortex-mixing and centrifugation at 12,000 × *g* for 5 min, the aliquot of the supernatant (20 μl) was injected onto HPLC system.

2.4. HPLC analysis

The chromatographic system consisted of a Nanospace SI-2 pump, a Nanospace SI-2 UV detector, a SI-2 autosampler and a S-MC system controller (Shiseido, Tokyo, Japan). The separation was performed on a Luna C8 column (3 μm, 2 mm i.d. × 100 mm, Phenomenex, Torrance, CA, USA) using a mixture of acetonitrile–ammonium formate (10 mM, pH 3.0) (35:65, v/v) at a flow rate of 0.2 ml/min. The column temperature was 30 °C and the detection wavelength was 340 nm.

2.5. Method validation

Batches, consisting of three calibration standards at each concentration, were analyzed on three different days to complete the method validation. In each batch, QC samples at 0.75, 2.50 and 7.50 μg/ml were assayed in sets of six replicates to evaluate the intra- and inter-day precision and accuracy. The percentage deviation of the mean from true values, expressed as relative error (RE), and the coefficient of variation (CV) serve as the measure of accuracy and precision. The limit of quantitation (LOQ) for butein was determined by a signal-to-noise ratio of >5:1.

The absolute recoveries of butein were determined by comparing the peak area of six extracted samples at the concentrations of 0.75, 2.50 and 7.50 μg/ml with the mean peak area of recovery standards. Three replicates of each of the recovery standards were prepared by adding butein standard to blank rat serum extracts.

To evaluate the three freeze/thaw cycle stability and room temperature matrix stability, six replicates of QC samples at each of the low and high concentrations (0.75 and 7.50 μg/ml, respectively) were subjected to three freeze/thaw cycles or were stored at room temperature for 12 h before processing, respectively. Six replicates of QC samples at each of the low and high concentrations were processed and stored under autosampler conditions for 24 h were assayed to assess post-preparative stability.

2.6. Animal study

The developed HPLC assay method was used in a pharmacokinetic disposition study after i.v. injection of butein to Sprague–Dawley rats (7–8 weeks of age,

body weight 229 ± 10 g, Biogenomics, Seoul, Korea). Animals were kept in plastic cages with free access to standard rat diet (Samyang, Seoul, Korea) and water. The animals were maintained at a temperature of 22–24 °C with a 12 h light/dark cycle and relative humidity of 50 ± 10%. The rats were anesthetized by i.p. injection of ketamine and xylazine (90:10 mg/kg) and cannulated with polyethylene tubing (0.58 mm i.d. and 0.96 mm o.d., Natume CO., Tokyo, Japan) in the left femoral and right jugular veins. After a 2-day recovery period, butein dissolved in saline was injected intravenously at a dose of 5 mg/kg into the left femoral vein of the rats ($n = 4$). Venous blood samples sere collected at 2, 5, 10, 15, 30 min, and 1, 2, 4, 6 and 8 h after i.v. injection. Blood samples were immediately centrifuged at 3000 g for 3 min and harvested serum samples were stored at −20 °C until analysis. Urine was collected over a 24 h period and a portion was stored at −20°C until analysis. The volume of the serum and urine samples used in the analysis was 50 μl.

3. Results and discussion

3.1. LC condition

Narrow-bore reversed-phase HPLC was used for the analysis of butein from serum samples because of advantages such as lower solvent consumption and increased sensitivity. The asymmetry factor of butein increased as the pH of the mobile phase buffer was increased from pH 3 to 6.5, reaching a optimum value (1.08) at pH 3.0. The capacity factor of butein was 2.1 using acetonitrile–ammoium formate (10 mM, pH 3.0). Butein has the absorption maximum at 340 nm and the molar absorption coefficient (l/mol/cm) of 13,900 at λ_{max}. HPLC with UV detection at 340 nm provided the sensitivity and specificity for the determination of butein in serum samples.

The representative HPLC chromatograms of the extracted blank rat serum, rat serum spiked with butein (2.5 μg/ml) and a rat serum sample obtained 15 min after i.v. administration of butein (5 mg/kg) are shown in Fig. 2. The analysis of blank serum samples from ten different sources did not show any interference at the retention time of butein (3.8 min), confirming the specificity of the present method.

3.2. Method validation

This method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation [11]. Calibration curves were obtained over the concentration range of 0.1–10 μg/ml of butein in serum. Linear regression analysis with a weighting of 1 per peak area gave the optimum accuracy of the corresponding calculated concentrations at

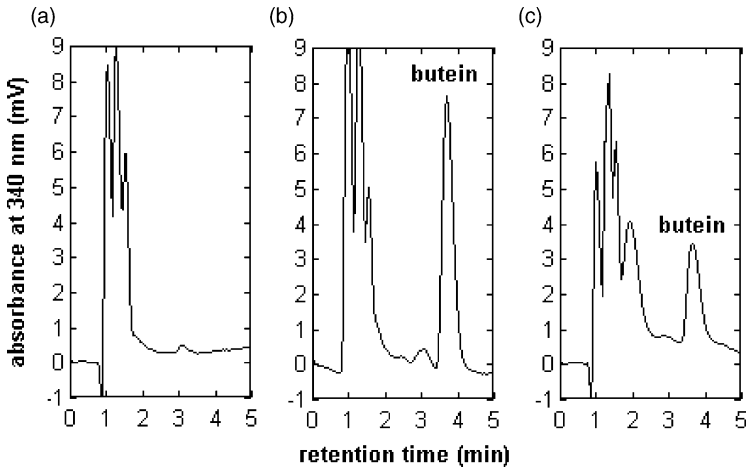

Fig. 2. HPLC chromatograms of (a) a blank rat serum and (b) rat serum sample spiked with 2.5 μg/ml of butein and (c) a rat serum sample obtained 15 min after i.v. administration of butein (5 mg/kg). Column: Luna C8 (3 μm, 100 mm × 2 mm i.d.); mobile phase: acetonitrile–ammonium formate (10 mM, pH 3.0) (35:65, v/v); flow rate: 0.2 ml/min; injection volume: 20 μl.

Table 1
Calculated concentrations of butein in calibration standards prepared in rat serum ($n = 9$)

	Theoretical concentration (μg/ml)						Slope	r^2
	0.10	0.50	1.00	2.00	5.00	10.0		
Mean (μg/ml)	0.10	0.46	0.91	2.12	5.01	10.3	38215	0.995
CV (%)	4.7	4.6	2.2	7.5	7.7	5.5	7.4	
RE (%)	4.5	−8.0	−9.4	5.8	0.1	3.3		

each level (Table 1). The low coefficients of variation (CV) value for the slope indicated the repeatability of the method (Table 1).

Table 2 shows a summary of intra- and inter-batch precision and accuracy data for QC samples containing butein. The precision of the method was determined as the CV of the calculated concentrations at each level. Both intra- and inter-assay CV values ranged from 2.7 to 7.5% at three QC levels. The accuracy of the method was measured by the percentage deviation of the mean from true values (RE). The inter- and inter-assay RE values for butein were −5.9 to 4.4% at three QC levels. These results indicated that the present method has the acceptable accuracy and precision. The LOQ was set at 0.1 μg/ml for butein using 50 μl of rat serum. CV and RE at the LOQ level were 7.5 and 4.4%, respectively (Table 2).

The extraction recoveries of butein from spiked rat serum were determined at the concentrations of 0.75, 2.50 and 7.50 μg/ml in six replicates. The recoveries of butein at 0.75, 2.50 and 7.50 μg/ml were 91.1 ± 3.3, 89.2 ± 2.2, and 89.4 ± 3.7%, respectively. The protein precipitation has been successfully applied to the sample preparation of butein from rat serum.

Stability of butein during sample handling (freeze-thaw and short-term temperature) and the stability of processed samples were evaluated (Table 3). Three freeze-thaw cycles and room temperature storage of the QC samples for 12 h before analysis, had little effect on the quantification. Extracted QCs and calibration standards were allowed to stand at ambient temperature for 24 h prior to injection without affecting the quantification.

Table 3
Stability of samples ($n = 6$)

Statistical variable	Theoretical concentration (μg/ml)	
	0.75	7.50
Three freeze and thaw stability		
Mean	0.73	7.18
CV (%)	3.7	5.6
RE (%)	−2.7	−4.3
Short-term temperature stability (12 h at room temperature)		
Mean	0.72	7.21
CV (%)	5.4	4.6
RE (%)	−4.0	−3.9
Post-preparative stability (24 h at room temperature)		
Mean	0.71	7.33
CV (%)	4.3	4.9
RE (%)	−5.3	−2.3

3.3. Application of method

This method has been successfully used to the pharmacokinetic study of butein after a bolus i.v. administration of 5 mg/kg of butein in male Sprague–Dawley rats. The mean serum concentration of butein versus time curve is shown in Fig. 3. The pharmacokinetic parameters of butein were determined

Table 2
Precision and accuracy of butein in quality control samples

	Intra-batch ($n = 6$)				Inter-batch ($n = 18$)		
QC (μg/ml)	0.10	0.75	2.50	7.50	0.75	2.50	7.50
Mean (μg/ml)	0.10	0.71	2.44	7.55	0.74	2.45	7.34
CV (%)	7.5	3.8	6.6	2.7	7.5	7.1	6.0
RE (%)	4.4	−5.9	−2.4	0.6	−1.6	−2.0	−2.2

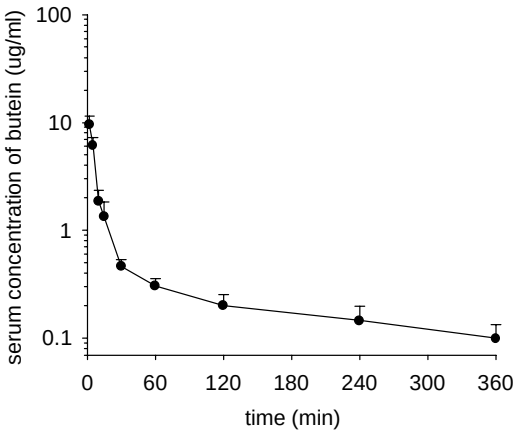

Fig. 3. Mean serum concentration-time plots of butein after an intravenous injection of butein (5 mg/kg) to four male rats. Each point represents the mean ± S.D.

Table 4
Pharmacokinetic parameters of butein obtained after intravenous injection (5 mg/kg) in male Sprague–Dawley rats ($n = 4$, mean ± S.D.)

$t_{1/2\lambda z}$ (h)	2.1 ± 0.8
AUC (μg min/ml)	145.6 ± 24.3
AUMC (μg min^2/ml)	8659.7 ± 6036.7
V_z (l/kg)	5.57 ± 1.15
C_{max} (μg/ml)	13.0 ± 6.2
Cl (ml/kg/min)	32.0 ± 6.8
Fe (%)	1.6 ± 1.4

by non-compartmental analysis (WinNonlin Scientific Consultants, NC, USA) and are shown in Table 4. Butein exhibited a high distribution volume ($V_z = 5569.3$ ml/kg) and systemic clearance (Cl = 32.0 ml/kg/min), and a moderate apparent elimination half-life ($t_{1/2\lambda z} = 2.1$ h). The urinary excretion was minimal (<1.6%).

In summary, a simple HPLC method was developed and validated for the analysis of butein in rat serum, using the protein precipitation as sample clean-up procedure. This assay method demonstrated the acceptable sensitivity, precision, accuracy, selectivity, recovery and stability with a small sample volume (50 μl) and a relatively short analysis time. This method was successfully applied to a pharmacokinetic study of butein in rats, where limited sample volumes were available.

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