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High-performance liquid chromatographic method for simultaneous determination of hawthorn active components in rat plasma

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Abstract

A simple HPLC method with photodiode-array (PDA) ultraviolet detection was developed for the simultaneous determination of four active polyphenol components of hawthorn (*Crataegus*), chlorogenic acid, epicatechin, hyperoside and isoquercitrin, in rat plasma. Following extraction from the plasma samples with ethyl acetate–methanol (2:1, v/v), these four compounds were successfully separated using a C_{18} column with a gradient elution of 5 and 25% acetonitrile in 25 m*M* phosphate buffer (pH 2.4). The flow-rate was set at 1 ml/min and the eluent was detected at 325 nm for chlorogenic acid, 278 nm for epicatechin, and 360 nm for both hyperoside and isoquercitrin. Narignin (0.82 μg) was used as the internal standard and was detected at 278 nm. The method is linear over the studied range of 0.16–40, 0.63–160, 0.13–32 and 0.13–30 μg/ml for chlorogenic acid, epicatechin, hyperoside and isoquercitrin, respectively. The correlation coefficient for each analyte was greater than 0.995. The intra-day and inter-day precision of the analysis was better than 4 and 7%, respectively. The extraction recoveries at low to high concentration were greater than 85% for both epicatechin and chlorogenic acid, and greater than 94% for both hyperoside and isoquercitrin. The detection limits were 0.04, 0.20, 0.03 and 0.03 μg/ml for chlorogenic acid, epicatechin, hyperoside and isoquercitrin. The developed method was used to analyze the plasma concentrations of the four analytes after the intravenous administration of hawthorn polyphenol extract to rats.

Keywords: Chlorogenic acid; Epicatechin; Hyperoside; Isoquercitrin; Hawthorn

1. Introduction

Hawthorn fruit refers to the bright red berries of *Crataegus* species. It has long been used as a folk medicine and is described by many pharmacopoeias. Pharmacological and toxicological studies have demonstrated that consumption of hawthorn fruit is associated with long-term medicinal benefits on the cardiovascular function [1]. Hawthorn extract has been confirmed by various studies to possess a wide range of pharmacological properties, such as sedative action [2], a protective effect against arrhythmia [3], an increase of coronary vessel flow [4], and a decrease of blood pressure and serum levels of

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cholesterol [5,6]. More recent studies [7,8] have indicated that phenolic compounds are probably the active constitutes of hawthorn fruits and contribute to the antioxidative effect. These compounds include flavonoids (hyperoside, isoquercitrin, quercitin, rutin), proanthocyanins (epicatechin) and phenolic acid (chlorogenic acid, protocatechic acid).

Although the discovery of hawthorn's health-promoting effects has been well described, there is a lack of information concerning the extent to which the active components are absorbed, and their pharmacokinetics and bioavailabilities. It is necessary to develop analytical methods for biological samples such as plasma, etc.

Several previous papers have described a reversed-phase HPLC method for the quantitative determination of the flavonoids of hawthorn [9–14]. These papers mainly focused on the analysis of rutin, quercitin, hyperoside, vitexin or vitexin derivatives in plant tissue. However, no method has been described for the simultaneous determination of chlorogenic acid, epicatechin, hyperoside and isoquercitrin (Fig. 1) in either plant tissue or biological fluids after consumption of hawthorn, even though they are the four major active components of hawthorn fruit.

In the current study, we developed a simple HPLC method for the simultaneous determination of the above four active components of hawthorn fruit in rat plasma using narigin (Fig. 1) as internal standard. This method takes advantage of photodiode-array (PDA) UV detection for measuring all analytes at various wavelengths in a single injection. This is suitable for pharmacokinetics studies of hawthorn components. In the present article, we also report the use of this method in determining the plasma levels of the four hawthorn components following intravenous administration of hawthorn polyphenol extract.

2. Materials and methods

2.1. Reagents and chemicals

Chlorogenic acid (ChA), epicatechin (EC) and naringin (4′,5,7-trihydroxyflavanoe 7-rhamnoglucoside, NG, which was used as internal standard) were purchased from Sigma (St. Louis, MO, USA). Hyperoside (quercetin-3-*O*-galactoside, HP) and isoquercitrin (quercetin-3-*O*-glucoside, IQ) were isolated from hawthorn fruits as described in Section 2.2. The purity of these standards (ChA, EC, HP, IQ and NG) was tested and found to be better than 99.2% by HPLC. Acetonitrile (HPLC grade), methanol and ethyl acetate (analytical grade) were obtained from Labscan (Labscan Asia, Thailand). All reagents

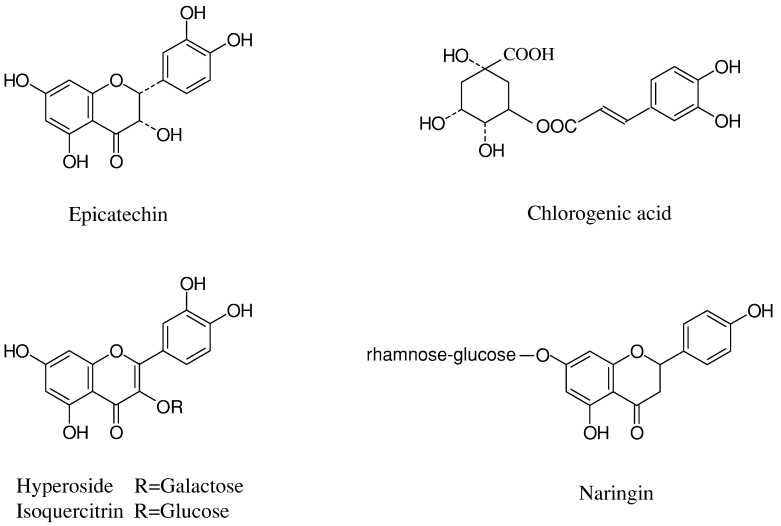

Fig. 1. Chemical structures of the four analytes, chlorogenic acid, epicatechin, hyperoside and isoquercitrin, and the internal standard, narigin.

were of analytical grade and used without further purification. Distilled and deionized water was used for the preparation of all solutions.

2.2. Isolation of hyperoside and isoquercitrin

One and a half kilograms of dried powder of hawthorn fruit without seeds (*Crateagus pinnatifida* Bge. var. *major* N.E.Br.) was extracted three times with 80% ethanol [8]. The pooled ethanol filtrates were concentrated in vacuum. The extract was dissolved in 4 L of water and extracted with 2 L of ether four times to remove the lipid components, and then extracted with 2 L of ethyl acetate four times. The ethyl acetate layer was evaporated to produce a final extract of 22.5 g. The extract was subjected to silica gel column chromatography and eluted with chloroform–methanol with increasing polarity. The fraction eluted with 10% methanol in chloroform was collected and further separated repeatedly on a Sephadex LH-20 column eluted with methanol to obtain HP (114 mg) and a mixture of HP and IQ. This HP and IQ mixture was further separated by HPLC using a C_{18} column (Radial-pak C_{18} cartridge, 10 cm×8 mm I.D.; 4 μm particle size, Waters, Milford, MA, USA) eluted with CH_3CN–H_2O (15:85, v/v) at a flow-rate of 3.5 ml/min to obtain IQ at 6.3 min and HP at 5.5 min. The total yields were 83.33 mg/kg for HP and 16.67 mg/kg for IQ. The chemical structures of both compounds were identified on the basis of their physicochemical properties and spectral data (mainly MS, ^{1}H- and ^{13}C-NMR) after comparison with published data [15,16].

2.3. Instruments

The liquid chromatographic system used was a Beckman series Gold HPLC system (Beckman Coulter, Fullerton, CA, USA) equipped with a 126 solvent delivery module, a 168 photodiode-array (PDA) UV detector and a 507e autosampler. System control and data analyses were carried out using Gold Nouvean software (revision 1.6, Beckman). The chromatographic separation of the four compounds and internal standard was achieved by using a reversed-phase HPLC column (Radial-pak C_{18} cartridge, 10 cm×8 mm I.D.; 4 μm particle size, Waters) protected by a precolumn filter (Nove-pak C_{18} Guard-pak, Waters).

2.4. Chromatographic conditions

The elution gradient for HPLC analysis consisted of two solvent compositions: 5% (solvent A) and 25% (solvent B) acetonitrile in 25 m*M* sodium phosphate buffer adjusted to pH 2.4 by concentrated phosphate acid. Gradient elution was carried out according to the following program: solvent B was increased from 10 to 80% in the first 20 min, held for 10 min and then returned to 10% in 5 min. The total run time was 35 min including equilibration of the system. The flow-rate was 1 ml/min. The eluent was monitored by a UV detector and the absorbance spectra (200–400 nm) were collected continuously during the course of each run. The detection wavelength was set at 278 nm for EC and NG, 325 nm for ChA, and 360 nm for HP and IQ. The sample injection volume was 100 μl.

2.5. Preparation of standards

Solutions of ChA, EC, HP and IQ at 2.00 mg/ml were prepared separately in 50% methanol. A stock solution containing ChA, EC, HP and IQ was prepared by mixing and diluting the above separate solutions with 50% methanol to yield concentrations of 0.20, 0.80, 0.16 and 0.16 mg/ml, respectively. The internal standard solution containing 41 μg/ml of NG was prepared in the same solvent. These solutions were stored at −20°C and were found to be stable for at least 12 months. Working standard solutions for spiking plasma were freshly prepared by diluting the stock solution in water at ratios of 1:1.25, 1:2.5, 1:5, 1:10, 1:20, 1:40, 1:80, 1:160 and 1:320, respectively.

The samples for plasma standard calibration curves were prepared by spiking the blank rat plasma (100 μl) with 25 μl of the appropriate working solution to yield the following concentrations: ChA at 0.16–40.00 μg/ml, EC at 0.63–160.00 μg/ml, and both HP and IQ at 0.13–32.00 μg/ml. Quality control samples used for the study of intra-day and inter-day accuracy and precision, extraction recovery and stability were prepared in the same way as the calibration samples. Specific quality control samples

representing low, middle and high concentration were 0.63, 5.00 and 20.00 μg/ml for ChA; 2.50, 20.00 and 80.00 μg/ml for EC; and 0.50, 4.00 and 16.00 μg/ml for both HP and IQ.

2.6. Calibration curves

The standard calibration samples were mixed with 20 μl of internal standard (41 μg/ml) and 25 μl of ascorbic acid water solution (20%), and then extracted as described in Section 2.7 below. Calibration curves were constructed by plotting the peak-area ratios of each analyte/internal standard versus analyte concentration in plasma. In order to avoid undue bias to the low concentrations of the standard curve by the high concentrations, the calibration curve of each compound was split into two ranges: 0.16–2.50 and 2.50–40.00 μg/ml for ChA; 0.63–10.00 and 10.00–160.00 μg/ml for EC; and 0.13–2.00 and 2.00–32.00 for both HP and IQ.

2.7. Extraction procedure

A 100 μl plasma sample was aliquoted in a glass centrifuge tube and spiked with 20 μl of internal standard (41 μg/ml) and 25 μl of ascorbic acid water solution (20%) to prevent oxidation [17,18]. The mixture was extracted once with 3 ml of EtOAc–MeOH (2:1, v/v) by vortex mixing for 1 min and centrifuged at 4000 rpm for 5 min to separate the protein from the organic phase. The supernatant was transferred to a clean glass tube and evaporated to dryness in vacuum by a centrifugal concentrator (Labconco, USA) at room temperature. The residue was reconstituted in 300 μl of 10% acetonitrile in sodium phosphate buffer (pH 2.4). After centrifuging at 13 000 rpm for 10 min, 100 μl of the supernatant was injected into the HPLC system for analysis.

2.8. Validation of the method

Validation of the HPLC method was performed by determining the intra-day, inter-day accuracy and precision, and percentage of recovery of the four analytes under the extraction and analytical condition. All procedures were performed as described in Section 2.7.

The quality control samples were analyzed in a set of five on a single assay day to determine intra-day precision and accuracy, and analyzed in duplicate on each of seven separate days to determine inter-day precision and accuracy. The extraction recovery was determined in sets of five by measuring the amount of each compound recovered after extraction.

The stability of the four analytes was determined in two ways. (1) The stability of analytes in the plasma sample stored at −80°C was determined in 1 month. (2) The stability of the analytes after extraction from plasma and dissolving in sodium phosphate buffer (pH 2.4) containing 10% acetonitrile for up to 48 h at room temperature. The quality control samples in triplicates at low, middle and high concentration were used.

2.9. Limit of detection

Blank rat plasma was spiked with decreasing concentrations of the four studied compounds and the sample was analyzed as described above. The limit of detection was defined as the lowest concentration of the drug resulting in a signal-to-noise ratio of 3:1.

2.10. Assay application

Hawthorn polyphenol extract was administered to five male Sprague Dawley rats intravenously with a bolus dose of 220 mg/kg, equivalent to 7.5, 34.8, 6.0 and 4.5 mg/kg of ChA, EC, HP and IQ, respectively. Venous blood samples were collected and centrifuged at 5, 10, 20, 40, 60, 90, 120, 180, 240 and 360 min for EC and at 2, 5, 10, 15, 20, 30, 40, 50, 60, 90 and 120 min for ChA, HP and IQ. The plasma sample (100 μl) was analyzed immediately or stored at −80°C until analysis.

3. Results and discussion

3.1. Chromatography

The purpose of this study was to develop a simple and rapid HPLC method for the simultaneous determination of active components of hawthorn in

biological fluid. Among the four analytes, HP and IQ have very similar chemical structures; i.e., 3-galactoside and 3-glucoside of quercetin, respectively. It is very difficult to separate them completely when elution is carried out in a single solvent system. In this study, a gradient elution of acetonitrile in sodium phosphate buffer at pH 2.4 was used to achieve complete separation. Fig. 2 shows representative HPLC profiles of a blank plasma sample (upper panels) and a blank plasma sample spiked with standard solution (lower panels) for ChA at 325 nm (left panel); EC and NG at 278 nm (middle panel); and HP and IQ at 360 nm (right panel). No interference was observed under the assay conditions. The peaks of the analyte in the plasma were identified by comparing their retention time with that of the standard and further confirmed by their on-line UV spectra. Typical retention times were as follows: ChA, 13.3 min; EC, 17.2 min; HP, 23.9 min; IQ, 24.4 min; and NG, 28.2 min. The total run time was 35 min, including equilibration.

3.2. Sample preparation

In this study, several mixtures of organic solvents were tested for extraction of the four analytes from rat plasma. They included EtOAc, EtOAc–MeOH (2:1, v/v), EtOAc–MeOH (1:1, v/v) and MeOH. The result (Table 1) showed that HP and IQ could be recovered in all the systems tested with a yield of >90%. However, the recovery of ChA and EC was only about 60% when extracted with EtOAc. The addition of MeOH increased the recovery to >80%. Since MeOH cannot be easily evaporated, the mixture with the least amount of MeOH (EtOAc–MeOH, 2:1) was chosen as the extraction solvent.

3.3. Linearity and limit of detection

Calibration curves of the test compounds were linear over the low and high concentration range with $r^2 > 0.995$ for all four analytes (Table 2). The

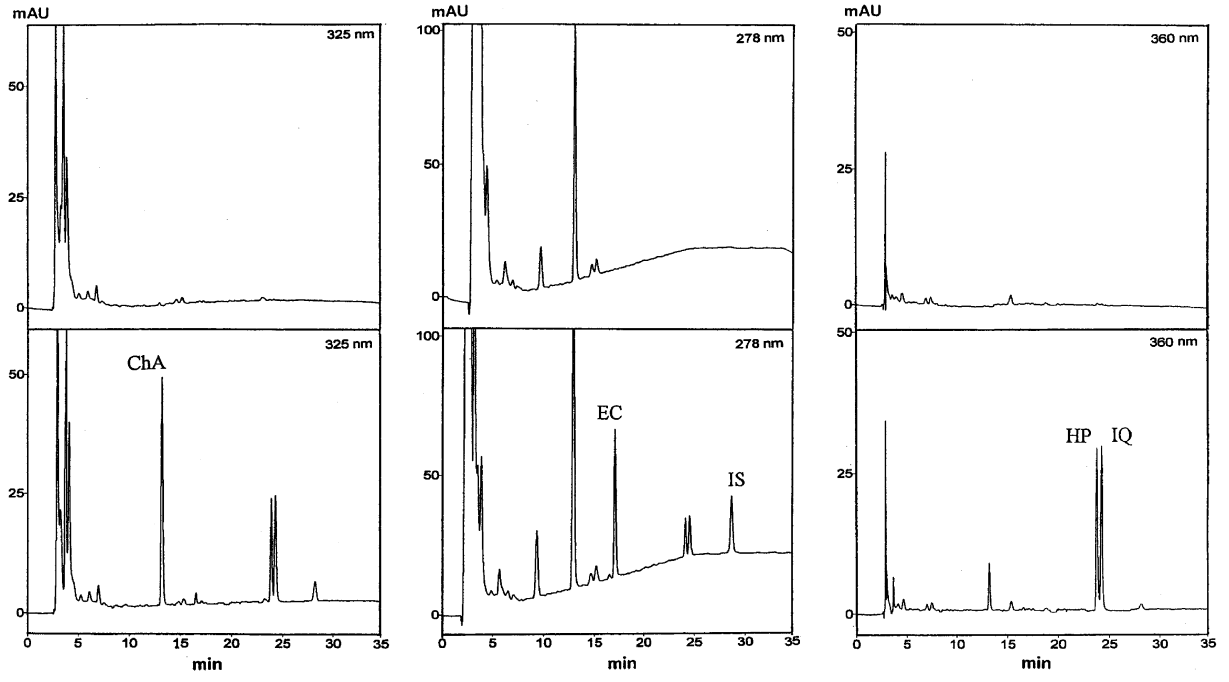

Fig. 2. HPLC chromatograms of a blank plasma sample (upper panels) and a blank plasma spiked with standards (lower panels) detected at 352 nm for chlorogenic acid (CA, 5 μg/ml) (left panel), 278 nm for epicatechin (EC, 20 μg/ml) and naringin (NG) used as internal standard (middle panel), and 360 nm for hyperoside (HP, 4 μg/ml) and isoquercitrin (IQ, 4 μg/ml) (right panel).

Table 1
Recovery values (% R, $n = 4$) for chlorogenic acid (ChA), epicatechin (EC), hyperoside (HP) and isoquercitrin (IQ) extracted from rat plasma using different solvents

Compound	Extract solution (EtOAc–MeOH, v/v)							
	1:0		2:1		1:1		0:1	
	% R	% RSD	% R	% RSD	% R	% RSD	% R	% RSD
ChA	62.25	1.70	92.00	2.15	92.00	0.15	86.75	0.57
EC	52.65	9.80	83.27	5.26	86.14	0.16	86.54	0.90
HP	93.20	0.68	101.11	5.46	100.89	2.21	101.83	0.62
IQ	94.11	2.96	100.50	0.93	97.83	2.14	102.67	1.92

The study was performed using a quality control sample containing 5.00, 20.00, 4.00 and 4.00 μg/ml of ChA, EC, HP and IQ, respectively.

Table 2
Linearity and sensitivity of detection for chloragenic acid (ChA), epicatechin (EC), hyperoside (HP) and isoquercitrin (IQ) in rat plasma

Analyte	Range studied (μg/ml)	Correlation coefficient (r^2)	Sensitivity (μg/ml)
ChA	0.16–2.50	0.9993	0.04
	2.50–40.00	0.9998	
EC	0.63–10.00	0.9946	0.20
	10.00–160.00	0.9997	
HP	0.13–2.00	0.9998	0.03
	2.00–32.00	0.9995	
IQ	0.13–2.00	0.9975	0.03
	2.00–32.00	0.9993	

detection limit for ChA, EC, HP and IQ in plasma was determined to be 0.04, 0.20, 0.03 and 0.03 μg/ml, respectively, with $S/N = 3{:}1$ (Table 2).

3.4. Accuracy, precision and recovery

Analytical accuracy and precision data are shown in Table 3 and are expressed as mean detected concentration and relative standard deviation (RSD %). The precision of the four analytes at low to high concentrations was better than 4 and 7% for intra-day and inter-day assays, respectively.

Table 3
Intra-day and inter-day (7 separate days) accuracy and precision and extraction recovery for the determination of chloragenic acid (ChA), epicatechin (EC), hyperoside (HP) and isoquercitrin (IQ) in rat plasma

Analyte	Nominal conc. (μg/ml)	Intra-day ($n = 5$)		Inter-day ($n = 14$)		Mean recovery (%) ($n = 5$)	RSD (%)
		Mean detected conc. (μg/ml)	RSD (%)	Mean detected conc. (μg/ml)	RSD (%)		
ChA	0.63	0.66	2.03	0.67	5.06	85.56	2.98
	5.00	4.95	1.51	5.01	3.82	91.35	0.52
	20.00	20.01	1.49	20.26	2.56	94.92	1.20
EC	2.50	2.42	2.30	2.46	5.18	85.19	2.17
	20.00	20.81	0.51	20.33	3.14	89.97	1.53
	80.00	80.20	1.19	80.88	2.06	88.87	1.48
HP	0.50	0.54	3.07	0.47	4.17	93.94	3.23
	4.00	4.02	1.99	4.20	3.55	99.24	2.00
	16.00	16.05	1.55	15.98	1.39	99.76	1.56
IQ	0.50	0.55	2.98	0.45	6.37	96.91	4.59
	4.00	4.01	2.02	4.22	3.72	97.32	1.98
	16.00	16.10	1.25	16.01	2.24	101.11	1.49

RSD, relative standard deviation.

The recovery was calculated by comparing the respective peak area of the extracted sample relative to that of the unextracted standard containing an equivalent amount of the standard. The extraction recoveries of the four analytes ($n = 5$) from spiked rat plasma (Table 3) were satisfactory at low, middle and high concentrations. They varied from 86 to 95% for ChA, from 85 to 90% for EC, from 94 to 100 for HP and from 97 to 101% for IQ. Recovery of the internal standard was very consistent with a mean of 93.57% and a RSD of 2.09% ($n = 20$).

3.5. Stability

The four analytes were stable in plasma samples stored at −80°C for at least 1 month (Fig. 3). The four analytes were also stable in plasma extract dissolved in sodium phosphate buffer (pH 2.4) containing 10% acetonitrile for up to 48 h (Fig. 4), demonstrating the stability of the analytes when placed in the autosampler waiting to be analyzed.

3.6. Application

This new HPLC method was successfully applied to estimate the plasma concentration of the four hawthorn compounds in rat. Fig. 5 shows the plasma concentration–time profiles of ChA, EC, HP and IQ in rat following intravenous administration of hawthorn extract at a dose of 220 mg/kg, equivalent to 34.8, 7.5, 6.0 and 4.5 mg/kg of epicatechin, chlorogenic acid, hyperoside and isoquercitrin, respectively.

4. Conclusion

This paper describes the validation of a HPLC method for the quantitation of the plasma concentrations of four active components of hawthorn: chlorogenic acid, epicatechin, hyperoside and isoquercitrin. Naringin was used as the internal standard. This method is not only simple and efficient with excellent accuracy, precision and reproducibil-

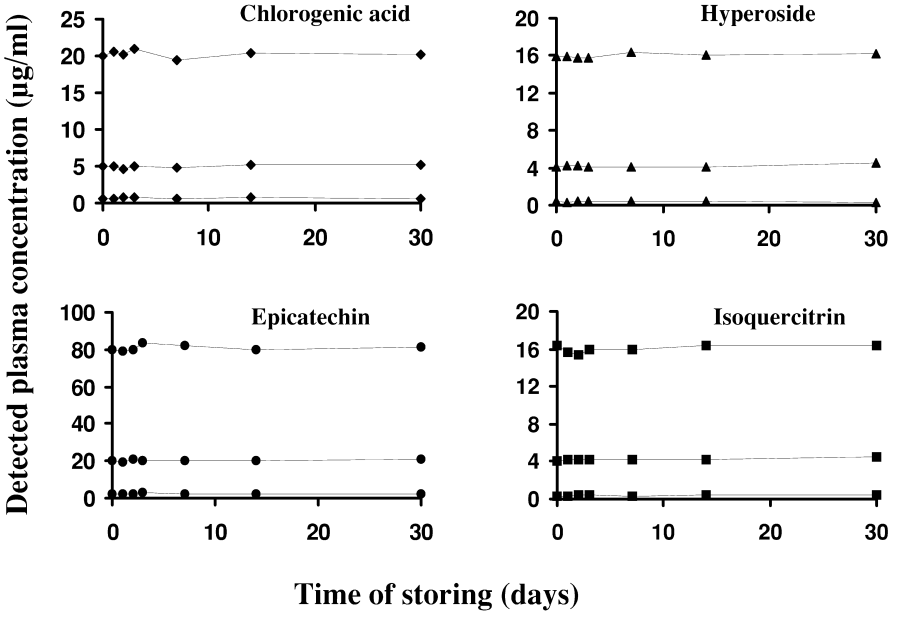

Fig. 3. Stability of chlorogenic acid (0.63, 5 or 20 μg/ml, ♦), epicatechin (2.5, 20 or 80 μg/ml, ●), hyperoside (0.5, 4 or 16 μg/ml, ▲) and isoquercitrin (0.5, 4 or 16 μg/ml, ■) in a rat plasma sample stored at −80°C. Data are expressed as the mean of three samples.

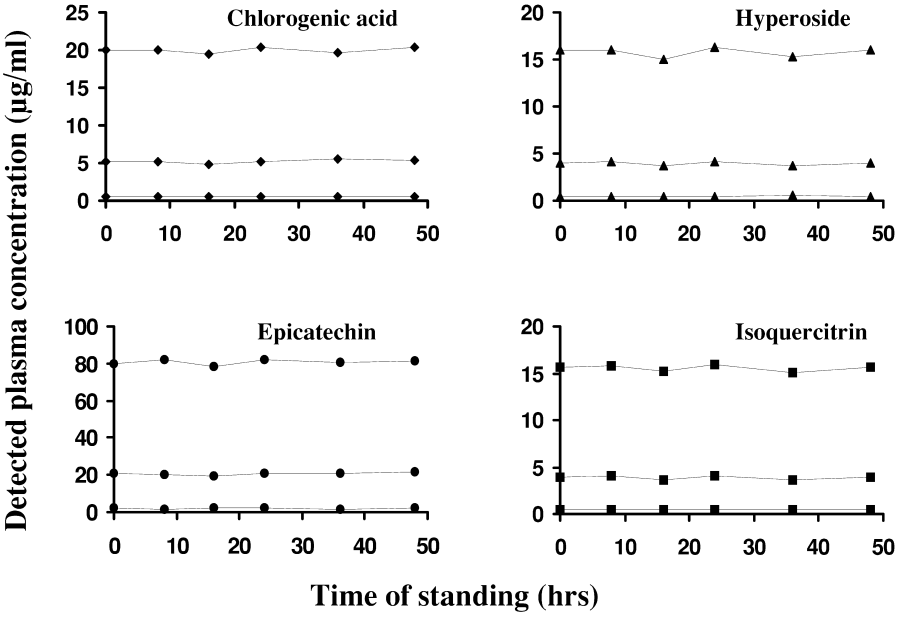

Fig. 4. Stability of chlorogenic acid (0.63, 5 or 20 μg/ml, ♦), epicatechin (2.5, 20 or 80 μg/ml, ●), hyperoside (0.5, 4 or 16 μg/ml, ▲) and isoquercitrin (0.5, 4 or 16 μg/ml, ■) in plasma extract dissolved in sodium phosphate buffer (pH 2.4) containing 10% acetonitrile. Samples were placed in the HPLC autosampler at room temperature and injected at time 0, 8, 16, 24, 36 and 48 h. Data are expressed as the mean of three samples.

ity, but also allows for the simultaneous determination of the four compounds and can be used for pharmacokinetic studies.

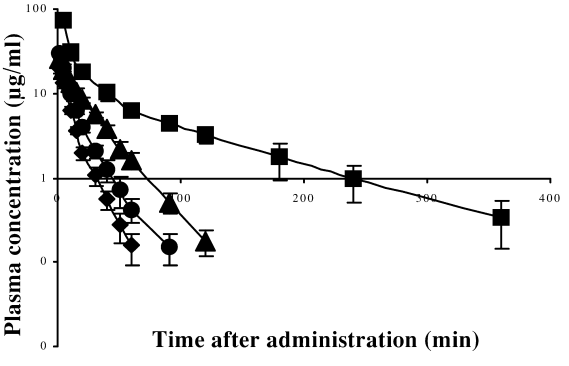

Fig. 5. Plasma concentration versus time profiles of epicatechin (■), chlorogenic acid (▲), hyperoside (●) and isoquercitrin (♦) in rats ($n = 5$) following intravenous administration of hawthorn extract at a single dose of 220 mg/kg, containing 34 mg of epicatechin, 8 mg of chlorogenic acid, 6 mg of hyperoside and 5 mg of isoquercitrin.

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