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Journal of Chromatography B, 665 (1995) 383–389

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of (+)-catechin in plasma by high-performance liquid chromatography using fluorescence detection

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First received 26 July 1994; revised manuscript received 16 November 1994; accepted 17 November 1994

Abstract

A high-performance liquid chromatographic method, using fluorescence detection, was developed for the determination of (+)-catechin in rabbit plasma. The procedure involved the precipitation of plasma protein using acetonitrile, followed by solid-phase adsorption onto alumina. After washing with water and methanol, the residue was vortex-mixed with perchloric acid solution to release the adsorbed (+)-catechin. Separation was performed on a reversed-phase column using an eluent consisting of phosphoric acid solution with 12% acetonitrile. The excitation and emission wavelengths were set at 280 and 310 nm, respectively. The retention times for (+)-catechin and the internal standard (deoxyhigenamine) were 6.87 and 8.47 min respectively, without any interference. Validations of accuracy and precision were satisfactory in both within- and between-run assays. All coefficients of variance were less than 6% and mean relative errors were within ± 3.75%. The average recovery was 73.77%. The limit of detection and quantitation were 1 ng and 0.02 μg/ml, respectively. Application of this method was successfully assessed by intravenous administration of a 15 mg/kg dose of (+)-catechin in rabbits. This new method provides a simple, specific and sensitive determination for (+)-catechin in rabbit plasma and is suitable for pharmacokinetic studies.

1. Introduction

(+)-Catechin (3,3′,4′,5,7-tetrahydroxyflavan), a naturally occurring flavonoid, has been shown to have cytoprotective properties in the treatment of acute viral hepatitis and alcohol-induced liver damage [1–3]. Inhibitory effects of (+)-catechin on chemically-induced carcinogenesis and mutagenesis have also been reported [4–9]. In some European countries, this drug is widely used, especially in the prevention of alcoholic hepatitis.

Many analytical methods are available for the determination of (+)-catechin, such as spectrophotometry utilizing the formation of colored species, difference spectra spectrophotometry, HPLC, etc. [10–12]. Most of these methods have been used in the analysis of (+)-catechin in food and plants. However, few methods can be used for analysis of the drug in biological fluids. In the papers of Hackett et al. [13] and Shaw et al. [14], (+)-catechin in human plasma was detected by an HPLC method using UV detection. However, this method is complex and the sensitivity is not high enough for pharmacokinetic studies. Pan et al. [15] also reported an HPLC method, involving solid-phase extraction by Sep-Pak C_{18} for sample preparation. This UV detection method

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SSDI 0378-4347(94)00535-4

used 1-ml samples of rabbit plasma and gave excellent recovery, but was still insufficiently sensitive and specific for detailed pharmacokinetic studies. Therefore, the aim of the present study was to develop a simple, accurate and sensitive HPLC method using fluorescence detection for the analysis of low levels of (+)-catechin in rabbit plasma to be used in pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

(+)-Catechin (Fig. 1) was purchased from Sigma (St. Louis, MO, USA). Deoxyhigenamine (DOHG; Fig. 1), the internal standard, was a gift from Dr. C.M. Chen (professor of the School of Pharmacy, Taipei Medical College). Methanol and acetonitrile were both HPLC-grade solvents obtained from BDH (Poole, UK). Alumina was obtained from Bioanalytical Systems (West Lafayette, IN, USA). Tris buffer, composed of tris(hydroxymethyl)aminomethane (Trizma base) and EDTA (disodium salt), was purchased from Sigma. Phosphoric acid (85%) and all other reagents were of analytical grade and purchased from Merck (Darmstadt, Germany).

2.2. Instrumentation and chromatographic conditions

The HPLC system consisted of an IRICA Model Σ-871 pump (Kyoto, Japan), a Shimadzu SIL-9A autosampler, a Shimadzu RF-530 fluorescence detector, and a Shimadzu C-R4A integrator (Kyoto, Japan). The column was a Cosmosil-AR column (C_{18}, 5 μm, 150 × 4.6 mm I.D., Nacalai Tesque, Kyoto, Japan). Fluorescence was monitored at an excitation wavelength of 280 nm and an emission wavelength of 310 nm.

The mobile phase was 12% acetonitrile in a 35 m*M* phosphoric acid solution (pH 2.5) which was filtered and degassed before use. Chromatography was performed at ambient temperature and the flow-rate of the mobile phase was 1.0 ml/min.

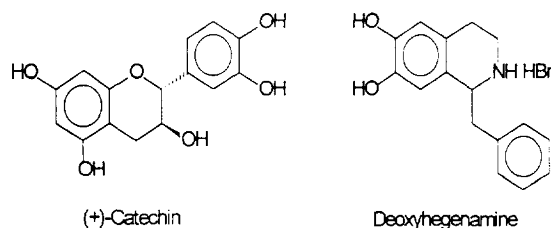

Fig. 1. Structures of (+)-catechin and the internal standard deoxyhigenamine.

2.3. Stability studies

To enable accurate quantitation of biological samples, the degradation of (+)-catechin in various pH buffer solutions at an ionic strength of 0.1 was studied first. The initial concentration of (+)-catechin was 0.01 m*M*. After incubation for a suitable time at 37°C, 20 μl of the solution was removed. The amount of (+)-catechin remaining was then analyzed by HPLC to determine the pH-dependent degradation rate.

The stability of (+)-catechin in blood and plasma was also examined. To 10 ml of either blank rabbit blood or plasma, (+)- catechin was added to a final concentration of 0.01 m*M*. The samples were incubated at 25°C or in an ice bath at 0°C. Subsequently, 250 μl of plasma was removed either directly, or after centrifugation of blood. The plasma was treated according to the same procedure as used for sample preparation (see below). After HPLC analysis, the degradation half-life of (+)-catechin was calculated according to first order kinetics.

2.4. Sample preparation

Plasma samples [25 μl mixed well with 225 μl of blank plasma for the first three plasma samples taken after intravenous injection of (+)-catechin or with 250 μl of plasma for the other samples] was thawed on an ice bath. Acetonitrile (0.5 ml) containing 0.8 μg of DOHG as the internal standard, was added. Following vortex-mixing for 1 min, the mixture was centrifuged at

10 000 g for 5 min and the supernatant was transferred to a glass tube containing 50 mg of alumina. A 1-ml aliquot of Tris buffer was then added. After vortex-mixing for 5 min and centrifugation, the residue was washed separately with 2 ml each of deionized water and then methanol. Finally, the residue was vortex-mixed with 250 μl of 0.25 M perchloric acid solution for 5 min and 125 μl of the supernatant was injected onto the HPLC system.

2.5. Calibration curve

Calibration samples were prepared fresh for each assay by mixing an adequate volume (ranging from 5 to 20 μl) of the (+)-catechin working solution [diluted from a stock solution which was prepared by dissolving (+)-catechin in pH 3 buffer solution with a concentration of 1 mg/ml] with 250 μl of blank rabbit plasma to obtain a calibration curve with concentrations ranging from 0.02 to 8 μg/ml. All samples were placed in an ice bath immediately after spiking and then analyzed according to the procedure described for sample preparation. Calibration graphs were constructed by linear regression of the peak-area ratio vs. concentration. No weighting process was used. To minimize errors due to analysis, a low calibration range from 0.02 to 0.4 μg/ml was calculated separately and used to calibrate appropriate samples. The mean relative errors of the calibration curve were also examined for validation.

2.6. Accuracy, precision and recovery

The accuracy and precision of the method were assessed by within- ($n = 6$) and between-run ($n = 18$) validations. Control samples were freshly spiked with the corresponding amount of (+)-catechin to provide final concentrations of 0.060, 0.32, 1.6, and 4.8 μg/ml. During each assay run, the control samples were positioned between the plasma samples and calibration samples. The control samples were also treated by the same procedure as described for sample preparation. By substituting the peak-area ratio into the calibration curve from the same run, the measured concentration could be obtained. By comparing calculated and theoretical concentrations, the relative errors and coefficients of variance could be obtained.

Recovery was assessed by comparing the peak-area ratio for extracted plasma samples ($n = 5$) spiked to 0.030, 0.060, 0.32, 1.6 and 4.8 μg/ml of (+)-catechin to the mean peak-area ratio for the equivalent standard injections ($n = 5$) after HPLC determination. The plasma samples were treated according to the procedure for sample preparation except that no internal standard was added. The equivalent standards were prepared by adding adequate amounts of (+)-catechin in 250 μl of perchloric acid (0.25 M) for comparison with the extracted samples. To all samples 0.8 g of DOHG (internal standard) was added and 125-μl aliquots of the final mixtures were injection onto the HPLC system. The peak-area ratio of (+)-catechin to DOHG was used to assess the efficiency of extraction.

2.7. Animal studies

Eight male, healthy New Zealand rabbits were used in the pharmacokinetic studies. They were fasted for 24 h prior to the experiment. A 15 mg/kg dose of (+)-catechin, dissolved in 2 ml of normal saline, was injected intravenously into the marginal ear vein of the rabbit. Blood samples (1 ml) were collected from the other marginal vein at 3, 6, 9, 15, 30, 45, 60, 80, 100, 120, 150, 180 and 240 min and placed immediately in an ice bath. The blood samples were centrifuged sequentially at 10 000 g for 3 min. Aliquots (250 μl) of plasma were then transferred to 2.2-ml microtubes and immediately stored at −80°C until analysis.

3. Results and discussion

3.1. Stability

Because of the reactivity of the catechol structure, the stability of (+)-catechin at various pHs was first assessed. As Fig. 2 shows, (+)-catechin was stable between pHs 2.0 and 5.0. In buffer

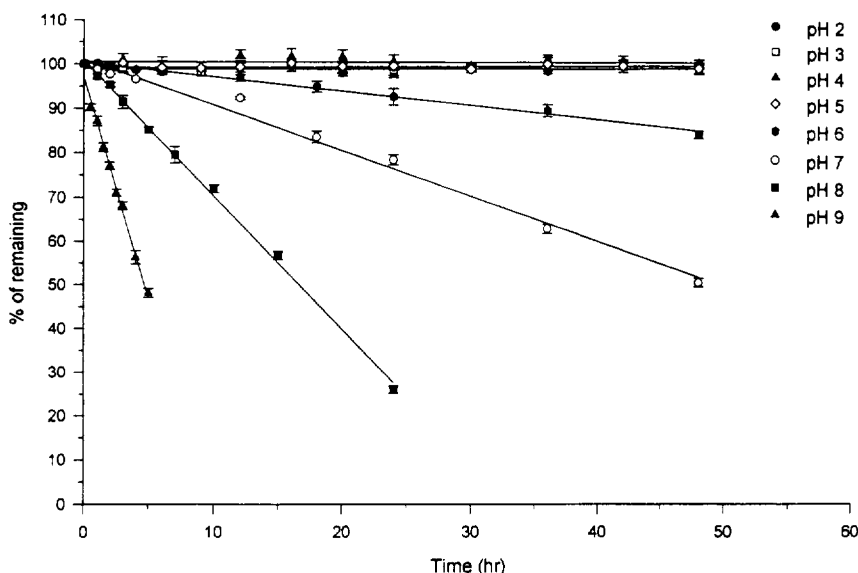

Fig. 2. pH-Dependent degradation profile of (+)-catechin at 37°C. Initial concentration of (+)-catechin 0.01 m*M*, ionic strength 0.1 ($n = 3$).

solutions at pH values above 5, (+)-catechin degraded rapidly with a half-life ranging between 3.5 and 198.6 min. These results indicate that biological samples containing (+)-catechin should be stabilized before analysis. The antioxidant activity of sodium sulfite and EDTA did not improve the stability of (+)-catechin. However, adjustment of the pH to values between 2.0 and 5.0 to stabilize the sample could not be used because this would interfere with the chelation of (+)-catechin on the alumina during extraction. Therefore, the stability of (+)-catechin in rabbit blood and plasma at temperatures of 0°C and 25°C was examined. As shown in Fig. 3, degradation of (+)-catechin in both rabbit blood and plasma was rapid at 25°C, and only 20% of the initial amount could be detected after incubation for 24 h at this temperature. However, after incubation at 0°C for up to 24 h, no degradation could be observed in plasma and 90% of the initial amount was recovered from the plasma after incubation of whole blood. Based on these results, it is important to keep the biological samples at a temperature as low as possible until analysis. In this study, blood samples were immediately placed in an ice bath and the plasma was stored frozen at −80°C until analysis.

3.2. Chromatography

Chromatograms of blank, spiked and sample plasma are shown in Fig. 4. No interfering peaks of endogenous compounds appeared and the approximate retention times for (+)-catechin and the internal standard were 6.87 and 8.47 min, respectively, with good resolution.

The analytical method described here consisted of adsorption of (+)-catechin on alumina followed by an acid-wash with perchloric acid solution. Using alumina as an adsorbent, (+)-catechin was successfully attracted to the hydroxyl groups of the alumina by hydrogen bonds or other molecular interactions under alkali conditions and released using perchloric acid solution after washing. Compared to the method reported by Pan et al. [15], the solid-phase adsorption used here was simpler and utilized a smaller amount of plasma. Fluorescence also provided a more specific and sensitive detection than UV. Based on a signal-to-noise ratio of 3,

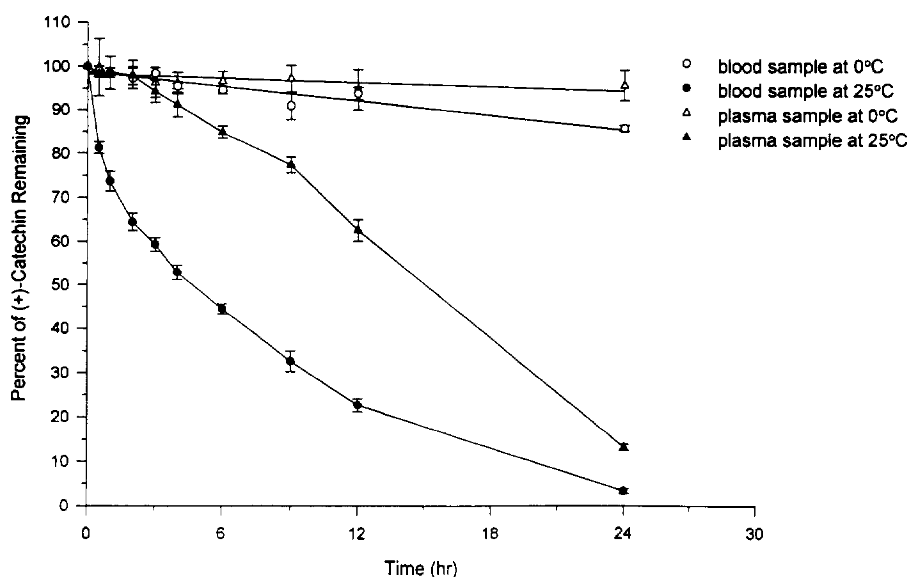

Fig. 3. Degradation of (+)-catechin in rabbit blood and plasma at 0°C and 25°C ($n = 3$).

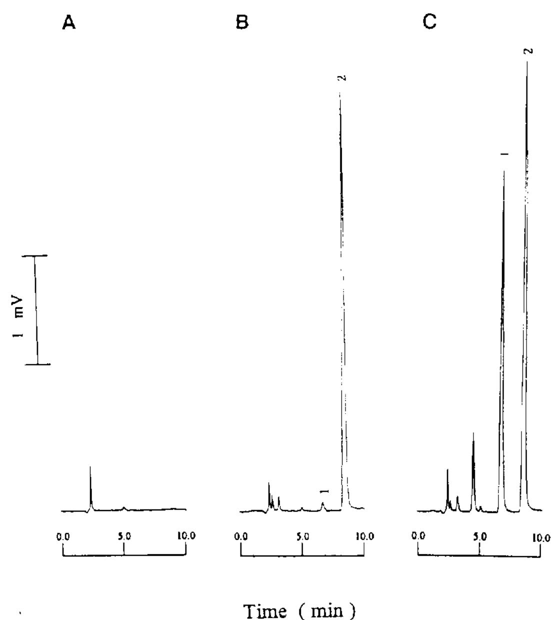

Fig. 4. Chromatograms of (A) blank plasma, (B) spiked plasma with 0.02 μg/ml of (+)-catechin and (C) a plasma sample taken 30 min after intravenous administration of 15 mg/kg of (+)-catechin with a calculated concentration of 1.333 μg/ml. All chromatograms were presented at the same mV range. Peaks: 1 = (+)-catechin, 2 = internal standard (DOHG).

the limit of detection was found to be 1 ng. The limit of quantitation was found to be 0.02 μg/ml.

Calibration curves for (+)-catechin were made using nine spiked plasma samples at concentrations of 0.02–8 μg/ml. Linearity was examined for all concentrations ($y = 0.4680x + 0.0067$, C.V. of slope was 1.77%, $n = 6$). For the lowest six concentrations (0.02–0.4 μg/ml), the linear regression for (+)-catechin in rabbit plasma was $y = 0.4751x + 0.0009$, and the C.V. of the slope was 3.10% ($n = 6$). R^2 was 0.999 for both concentration ranges. The mean relative errors ranged from −0.79 to 11.00% and the coefficients of variance were all less than 7%. These results indicate that this method is both stable and reproducible.

3.3. Accuracy, precision, and recovery

The accuracy and precision of the method were validated by within- and between-run assays. The results are shown in Table 1. At four different concentrations, the coefficients of variance were found to be less than 3% and the mean relative errors ranged from −3.75 to 1.71%. The accuracy and precision for between-run assays were also satisfactory. The coefficients

Table 1
Precision and accuracy validation of (+)-catechin in rabbit plasma

Spiked concentration (μg/ml)	Within-run			Between-run		
	Measured concentration (mean ± S.D., $n = 6$) (μg/ml)	Coefficient of variance[a] (%)	Relative error[b] (mean) (%)	Measured concentration (mean ± S.D., $n = 18$) (μg/ml)	Coefficient of variance[b] (%)	Relative error[b] (mean) (%)
0.060	0.061 ± 0.001	2.36	1.71	0.060 ± 0.003	5.19	−0.75
0.320	0.321 ± 0.007	2.27	0.17	0.322 ± 0.011	3.24	0.74
1.60	1.55 ± 0.020	1.27	−3.75	1.62 ± 0.056	3.48	1.06
4.80	4.75 ± 0.023	0.48	−0.11	4.83 ± 0.099	2.06	0.58

[a] Coefficient of variance = (S.D./mean) · 100%.
[b] Relative error = [(measured concentration − spiked concentration)/spiked concentration] · 100%.

of variance were less than 6% and the mean relative errors ranged from −0.75 to 1.06%. At a concentration of 0.02 μg/ml the coefficients of variance and related errors were 4.55 and 11% and at 8 μg/ml they were 0.38 and −0.79%, respectively. The % recoveries of (+)-catechin from rabbit plasma are listed in Table 2. The average recovery was 73.77%. From the analysis of the recovery samples, the coefficients of variance at the four highest concentrations were less than 7%. Only one coefficient of variance, at a concentration of 0.03 μg/ml, was over 10%.

3.4. Application in pharmacokinetic studies

The applicability of the method reported here was studied in rabbits given a 15 mg/kg dose of (+)-catechin by intravenous administration. The mean ($n = 8$) plasma concentration–time profile of (+)-catechin is shown in Fig. 5. The data were fitted to the two-compartment model of the PCNONLIN computer program [16], and the pharmacokinetic parameters were then obtained. The half-life for the elimination of (+)-catechin was 44 ± 6 min. The apparent volume of distribution and systemic clearance were 1.12 ± 0.14 l/kg and 0.080 ± 0.011 l/min/kg, respectively. The area under the plasma concentration curve was 159 ± 19 μg min/ml. These results disagree with those of Pan et al. [15] which could be due to the different analytical method and strain of rabbit used in our studies. As described above, our method is applicable to further basic pharmacokinetic studies on (+)-catechin.

Table 2
Recovery of (+)-catechin in rabbit plasma

Spiked concentration (μg/ml)	Peak-area ratio (mean ± S.D.)		Recovery (%) (mean ± S.D.) (%)	Coefficient of variance[a]
	Untreated	Treated with alumina		
0.030	0.014 ± 0.001	0.011 ± 0.001	74.13 ± 8.47	11.42
0.060	0.026 ± 0.001	0.020 ± 0.001	77.09 ± 4.98	6.46
0.320	0.143 ± 0.004	0.103 ± 0.001	71.65 ± 0.79	1.10
1.60	0.740 ± 0.021	0.531 ± 0.008	71.78 ± 1.13	1.57
4.80	2.164 ± 0.024	1.586 ± 0.036	73.26 ± 1.65	2.25

$n = 5$.
[a] Coefficient of variance = (S.D./mean) · 100%.

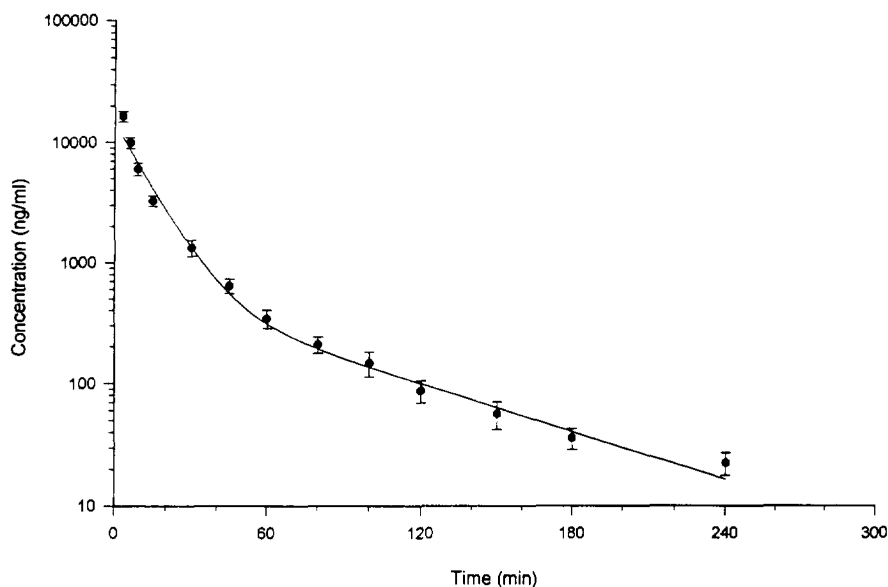

Fig. 5. Mean ± S.D. plasma concentration–time profile of (+)-catechin in rabbits ($n = 8$) after intravenous administration of a dose of 15 mg/kg of (+)-catechin.

4. Conclusions

Two novel ideas for the analysis of (+)-catechin in biological fluids are reported here. The first is the chelating adsorption of (+)-catechin onto alumina, and the second is the use of fluorescence detection in an HPLC system. This method provides a simple, specific, sensitive and reproducible method for the detection of (+)-catechin in rabbit plasma and can be successfully applied to pharmacokinetic studies.

Acknowledgement

This work was supported by a grant from Taipei Medical College (TMC-81-D106).

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