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Comparison of (+)-catechin determination in human plasma by high-performance liquid chromatography with two types of detection: fluorescence and ultraviolet

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Abstract

In this study we developed a high-performance liquid chromatography (HPLC) method for the determination of (+)-catechin in human plasma, using both fluorescence and UV detection. Sample preparation involved precipitation of plasma proteins using acetonitrile, followed by direct injection into the HPLC system using both types of detection. Validation of accuracy and precision were satisfactory for both within- and between-batch assays. For fluorescence detection, coefficients of variation were less than 6.47% and mean relative errors were within ±4.8%. The average recovery was 85.31%. The limit of detection and quantification were 5 ng/ml and 40 ng/ml, respectively. Ultraviolet detection was also used but appeared less sensitive and selective than fluorescence detection. This new method provides a simple, accurate, precise and specific method for the determination for (+)-catechin in plasma. © 1998 Elsevier Science B.V.

Keywords: Catechin

1. Introduction

(+)-Catechin (Fig. 1), a naturally occurring flavonoid, had been reported to be effective in the treatment of acute viral hepatitis [1]. More recently, some other in vitro studies have shown that this compound possesses a scavenger activity and inhibits low density lipoproteins oxidation [2,3]. The ability of (+)-catechin to act as antioxidant in the body initially depends upon its presence in plasma. However, few methods can be used for analysis of (+)-catechin in plasma [4–7]. Recently reported methods

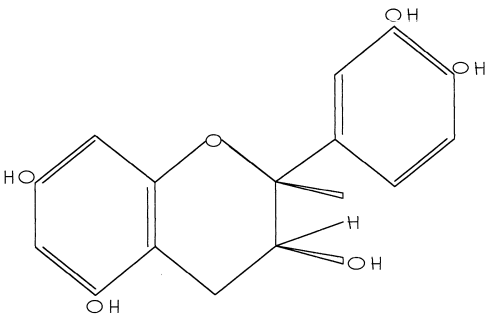

Fig. 1. (+)-Catechin.

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are complex to execute because sample preparation consists of extraction of the compound onto a resin; reversed C_{18} phase (Pan et al. [6], and Waterhouse et al. [4]) and alumina (Ho et al. [7]) have been used, followed by high-performance liquid chromatography (HPLC) separation. Both UV [4,6] and fluorescence [7] detection have been performed. The aim of the present study was to develop a simple, precise, accurate and sensitive method. We present a rapid procedure for sample preparation, avoiding successive plasma manipulations. Moreover, fluorescence and UV detection coupled with HPLC were both evaluated.

2. Experimental

2.1. Chemicals and reagents

(+)-Catechin was purchased from Aldrich (St. Quentin Fallavier, France). Methanol and acetonitrile were both HPLC grade and were obtained from Carlo Erba (Val de Reuil, France) and Merck (Nogent sur Marne, France), respectively. Orthophosphoric acid (85%) and ammonia were purchased from Panreac (Barcelona, Spain), and dihydrogen ammonium phosphate was obtained from Acros (Noisy le Grand, France).

2.2. Chromatographic conditions

A Hewlett–Packard, Model 1090 HPLC system with three low pressure pumps and a diode array UV detector coupled to an Hewlett–Packard Chemstation was used for solvent delivery and detection. A Shimadzu RF 530 fluorescence detector coupled with a Sefram recorder was also used for detection. A Hewlett–Packard column (Nucleosil 100 C_{18}, 250× 4 mm, 5 μm particle size) was used as the stationary phase. The solvents used for separation were: solvent A, 50 m*M* dihydrogen ammonium phosphate adjusted to pH 2.6 with orthophosphoric acid; solvent B, 20% A with 80% acetonitrile; and solvent C, 0.2 *M* orthophosphoric acid adjusted with ammonia to pH 1.5. Solvent gradient conditions (Lamuela-Raventos and Waterhouse [8]) are reported in Table 1. The column was thermostated at 25°C and the flow-rate was 0.7 ml/min. Fluorescence detection was monitored at an excitation wavelength of 280 nm and an emission wavelength of 310 nm, and UV detection was achieved at 280 nm.

2.3. Sample preparation

A 0.5 ml aliquot of the plasma sample was placed in an ice bath. Acetonitrile (0.7 ml) was added to effect protein precipitation. The mixture was then centrifuged at 2000 *g* and +4°C for 4 min. The supernatant was taken into a 1 ml syringe and filtered through a 0.5 μm pore size membrane (Millex-FH13; Millipore, St. Quentin Yvelines, France). A 25 μl volume of the filtered supernatant was directly injected into the HPLC system.

A stock solution of (+)-catechin was prepared by dissolving 20 mg of the compound in 10 ml of methanol. It was stored at +4°C, and diluted with methanol before use. The stock solution was stable for at least 1 month at +4°C.

2.4. (+)-Catechin calibration curve

Calibration samples were prepared before each assay by mixing the appropriate volume of (+)-catechin in 0.5 ml of water. Theses samples were analysed according to the procedure described for sample preparation. Calibration curves were con-

Table 1
Solvent gradient conditions (Lamuela-Raventos and Waterhouse [8])

Final time	A%	B%	C%
Initial	100	0	0
5	100	0	0
15	96	4	0
25	92	8	0
25.01	0	8	92
45	0	20	80
50	0	30	70
55	0	40	60
60	0	80	20
65	100	0	0

structed by linear regression of the peak-area ratio vs. concentration.

2.5. Plasma studies (accuracy, precision, recovery)

Plasma samples were freshly spiked with the corresponding amount of (+)-catechin to provide final concentrations of 0.05 to 32 μg/ml. During each assay run, a blank plasma sample was positioned between the plasma samples and calibration standards of (+)-catechin. The accuracy and the precision of the method were assessed by within (n=5) and between (n=5) analysis. Recovery was assessed by comparing the peak-area ratio for extracted plasma spiked to 0.045, 0.05, 0.150, 0.450 and 1.350 μg/ml to the mean peak-area ratio for the equivalent standard injection after HPLC determination with fluorescence detection. For UV detection, recovery was determined with the same method using plasma spiked to 3, 4, 8, 16 and 32 μg/ml of (+)-catechin.

2.6. Human study

This study was designed to determine whether (+)-catechin is present in human plasma after consumption of foods rich in flavonoids. Blood of five volunteers was drawn 12 h after consumption of a meal essentially composed of vegetables, fruits and 300 ml of red wine. Plasma was harvested by taking the blood samples into tubes containing EDTA as anticoagulant, followed by centrifugation at 1500 *g* for 20 min at +4°C. The plasma was held in an ice bath and was immediately analysed according to the described procedure.

2.7. Stability studies

To enable accurate quantification of the biological samples, the stability of (+)-catechin in plasma was studied. To 10 ml of blank plasma, (+)-catechin was added to a final concentration of 0.01 m*M*. The samples were incubated at −20, 0, and 25°C for 24 h. The plasma was treated according to the same procedure as was used for sample preparation, and analysed by HPLC. Long term stability at −20°C for 2 months was also investigated.

3. Results and discussion

3.1. Chromatography

Chromatograms of blank plasma and plasma spiked with (+)-catechin monitored by fluorescence detection are shown in Fig. 2. No interfering peaks of endogenous compounds appeared and the retention time of (+)-catechin was 42.5 min with good resolution. The limit of fluorescence detection was found to be 5 ng/ml and the limit of quantification was found to be 40 ng/ml. A chromatogram of plasma spiked with (+)-catechin monitored by UV is shown Fig. 3. The limits of detection and quantification with UV detection were found to be respectively, 1 μg/ml and 4 μg/ml.

3.2. Calibration curves

Calibration curves for (+)-catechin were constructed using samples at five concentrations of 0.045, 0.05, 0.15, 0.45 and 1.35 μg/ml for fluorescence detection and at 3, 4, 8, 16 and 32 μg/ml for UV detection. The respective linear regression equations were $y=0.902x+0.001$ ($r=0.999$) and $y=0.013x+1.857$ ($r=0.999$).

3.3. Accuracy, precision and recovery

The accuracy and precision of the method were validated on the basis of within and between run assay, for which the results are shown in Table 2. At four different concentrations, the coefficients of variation for the fluorescence method were found to be less than 3.9% and the mean relative errors ranged from −4.8 to 0.43%. The coefficients of variation and the mean relative errors for between run assay were also satisfactory with values less than 6.47% and ranging from −4.8 to 0.35% respectively. At four different concentrations, the coefficients of variation for the UV method were found to be less than 2.15% and the mean relative errors ranged from −0.75 to 0.9%. Values for between run assay were less than 2.63% and ranged from 0.33 to 2.6% respectively.

The linear regression equations for the assay were $y=0.995x-0.01$ ($r=0.999$; from 0.045 μg/ml to 1.35 μg/ml) for fluorescence method and $y=$

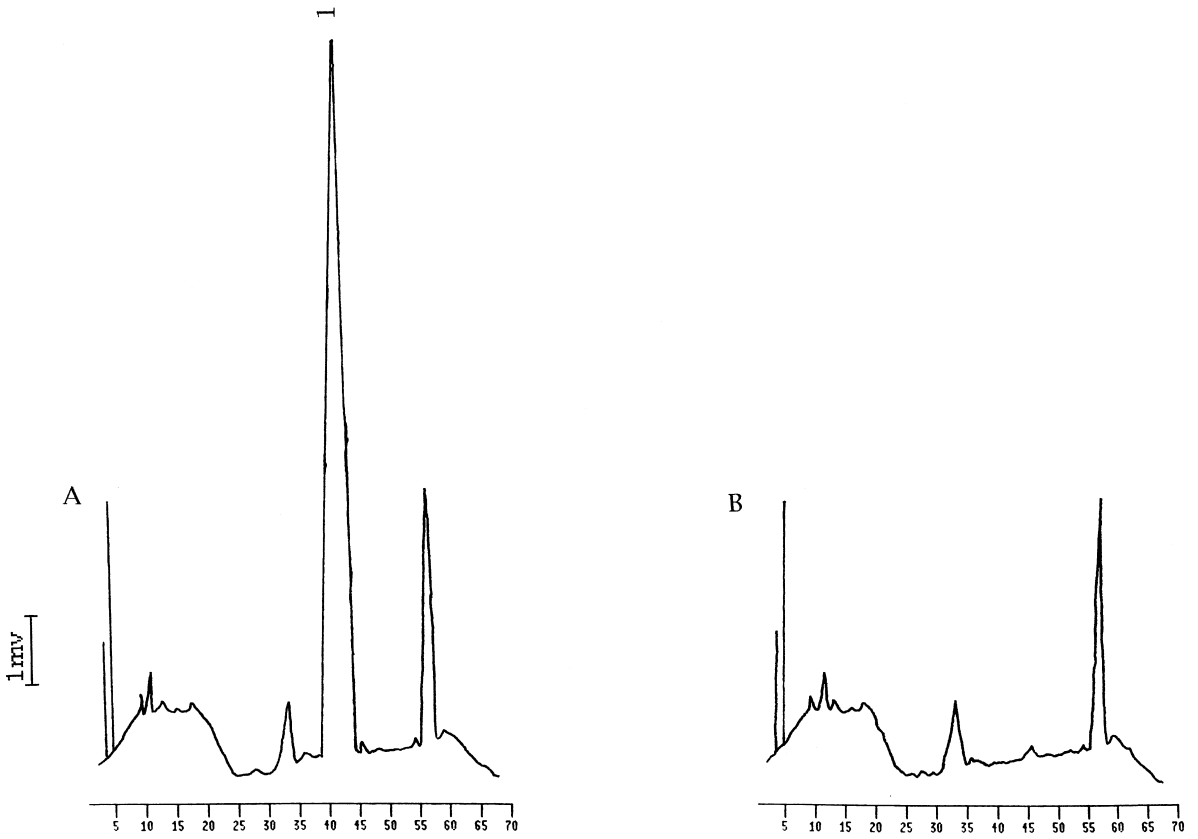

Fig. 2. Chromatograms of plasma spiked with (+)-catechin and blank plasma monitored by fluorescence: (A), chromatogram of spiked plasma with 1.35 μg of (+)-catechin; (B), blank plasma. Peak 1: (+)-catechin.

$0.013x+1.857$ ($r=0.999$; from 3 mg/ml to 32 mg/ml) for UV method.

The recovery of method was determined using both fluorescence and UV detection. The percent recoveries obtained for (+)-catechin are listed in Table 3. The average recoveries were respectively 85.31% and 84.87%. Based on the analysis of the recovery data, the coefficients of variation were found to be less than 5.2% and 3.5%.

3.4. Human study

Several human studies [4,5,9] on human absorption of (+)-catechin have reported values ranging between 0.1 to 3.4 μg of (+)-catechin per ml of plasma (Table 4). Therefore, this method coupled with fluorescence detection allows the determination of lower levels of (+)-catechin in human plasma, and therefore it is suitable for in vivo studies. However the limit of quantification of this method coupled with UV detection is too high, and does not permit its use in human studies. In order to use UV detection, prior concentration of plasma is required as already described by Pan et al. [6] and Waterhouse et al. [4]. Therefore, the detection of plasma samples was achieved by fluorescence. The plasma concentration of (+)-catechin of the five subjects at 12 h are reported in Fig. 4. Large variations (268 ng/ml to 809 ng/ml of plasma) were observed. Hence, significant amounts of (+)-catechin are present in blood circulation after consumption of a

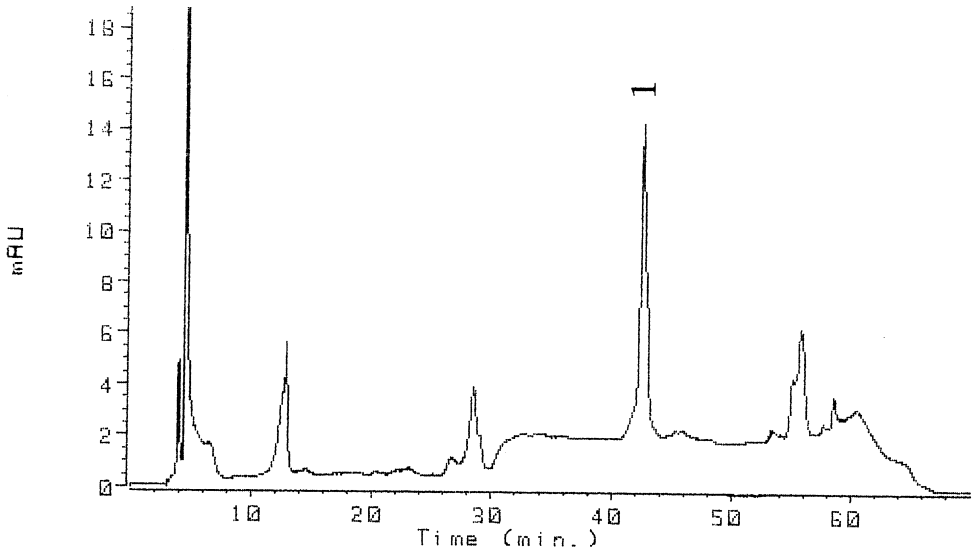

Fig. 3. Chromatograms of plasma spiked with 8 μg of (+)-catechin monitored by UV. Peak 1: (+)-catechin.

high level flavonoid diet. It will be interesting for further studies to determine the absorption of this compound after consumption of selected various types of diet, but it will be also interesting to determine if the presence of (+)-catechin in human blood is associated with increased protection against the oxidation of low density lipoproteins as observed by previous in vitro studies [2,3].

3.5. Stability

The stability of (+)-catechin in plasma was analysed at −20, 0, and 25°C (Fig. 5). Degradation was rapid at 25°C: only 8% of the initial amount could be detected after incubation for 24 h. After incubation at 0°C for the same time, 85% was recovered, and after incubation at −20°C, no degra-

Table 2
Precision and accuracy validation of (+)-catechin in human plasma for both fluorescence and UV detection

Spiked conc. (μg/ml)	Within-run measured conc. (mean±S.D, n=5) (μg/ml)	Coefficient of variance (%)	Relative error (mean) (%)	Between-run measured conc. (mean±S.D, n=5) (μg/ml)	Coefficient of variance (%)	Relative error (mean) (%)
Fluorescence detection						
0.050	0.047±0.002	3.9	−4.8	0.047±0.003	6.47	−4.8
0.150	0.149±0.004	2.66	−0.43	0.149±0.004	2.82	−0.53
0.450	0.451±0.004	0.97	0.3	0.452±0.005	1.07	0.35
1.350	1.350±0.002	0.2	0.1	1.351±0.004	0.32	−2.07
UV detection						
4	3.97±0.08	2.15	−0.75	4.10±0.10	2.63	2.6
8	8.07±0.15	1.86	0.9	8.07±1.97	1.97	0.87
16	16.08±0.14	0.85	0.6	16.09±0.14	0.86	0.56
32	32.10±0.27	0.84	0.37	32.10±0.26	0.82	0.33

Table 3
Recovery of (+)-catechin in human plasma for fluorescence and UV detection methods

Spiked concentration (μg/ml)	Recovery (mean ±S.D.) (%)	Coefficient of variance (%)
Fluorescence detection		
0.045	84.78±4.35	5.13
0.050	86.27±1.64	1.9
0.150	85.14±1.07	1.25
0.450	85.51±0.75	0.88
1.350	84.87±0.17	0.2
UV detection		
3	87.87±2.90	3.45
4	85.00±1.40	1.64
8	84.02±1.01	1.2
16	86.44±1.14	1.32
32	85.01±1.93	2.27

Table 4
Plasma level of (+)-catechin after oral administration

Study	Administrated dose (mg)	Plasma level (μg/ml)
Waterhouse et al. [4]	80 in wine	0.58 to 3.4
Hackett et al. [5]	2000 in water	0.1 to 1.5
Balant et al. [9]	2000 in water	2.3

dation was observed. After storage for 2 months at −20°C, 97% of the initial amount was recovered. Therefore, in this study, plasma samples were stored at −20°C until analysis and were placed in an ice bath during sample preparation.

4. Conclusion

This new method for the analysis of (+)-catechin combines the simplicity of protein precipitation with the sensitivity of fluorescence. The method is precise, accurate and sensitive and can be used to determine the absorption of (+)-catechin from the

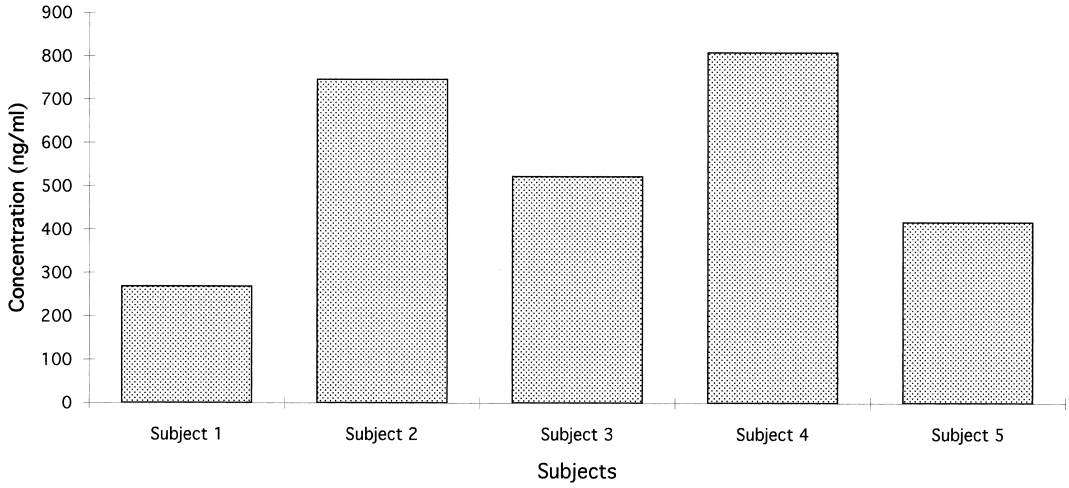

Fig. 4. Concentration of (+)-catechin in human plasma for five subjects.

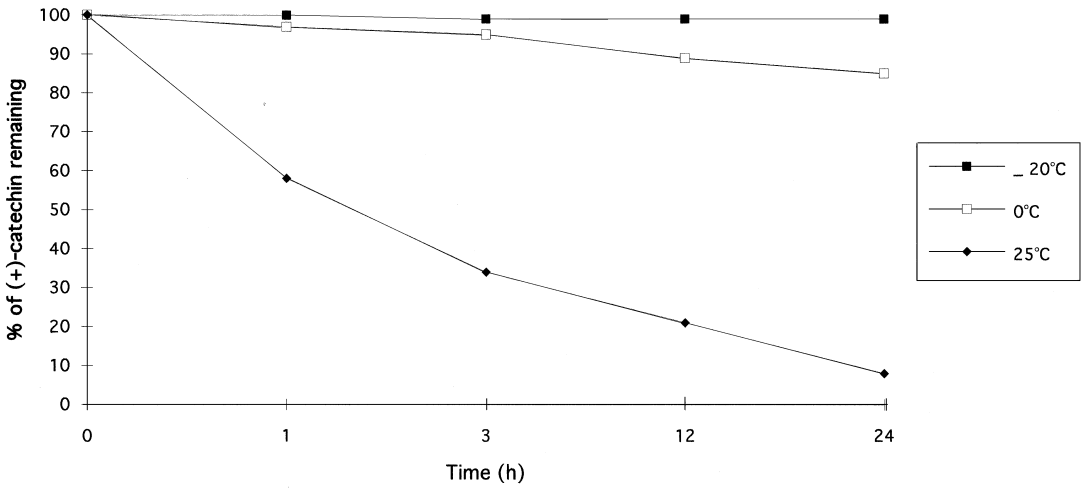

Fig. 5. Stability of (+)-catechin in plasma at −20, 0 and 25°C for 24 h.

diet. It will be interesting for future human studies to compare its absorption from various selected diets.

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