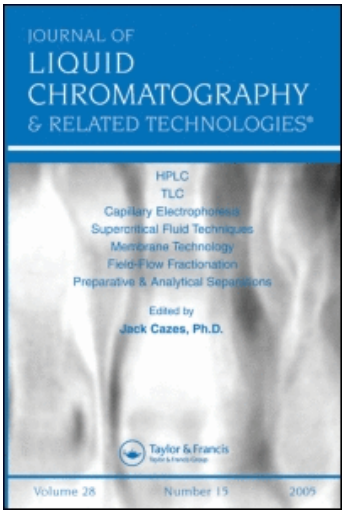

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Simple HPLC Method, with Fluorometric Detection, for Studying the Oral Absorption of Monomeric Catechins in a Small Animal Model

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Abstract: A high performance liquid chromatographic (HPLC) method, with fluorometric detection, was developed for studying the oral absorption and ensuing plasma pharmacokinetics of (+)-catechin and (−)-epicatechin in a small animal model. A plasma sample was first incubated with buffered β-glucuronidase plus sulfatase to release the catechins from their conjugated forms, and next deproteinized with methanol-perchloric acid (3 + 2) mixture containing an internal standard ((+)-catechin for the assay of (−)-epicatechin and the latter for the assay of the former). Analyses were carried out on a Microsorb-MV C18 column, with methanol-water-formic acid (15 : 84 : 1) flowing at 1 mL/min, and a fluorometric detector set at an excitation wavelength of 280 nm and an emission wavelength of 310 nm. (+)-Catechin and (−)-epicatechin eluted at 6.7 min and 13.8 min, respectively. Detector responses were linearly related to concentrations of catechin compound in the range 5–160 nM ($r^2 \geq 0.993$). The limits of detection were 0.75 nM for (+)-catechin and 1.5 nM for (−)-epicatechin. Accuracy and precision were evaluated at three concentrations of each catechin compound. Recoveries of (+)-catechin and (−)-epicatechin from spiked rat plasma ranged from 95.0 to 101.8% and from 98.7 to 102.5%, respectively. The RSD values for interday variability were in the range 1.27–4.07% for (+)-catechin and 0.47–2.04% for (−)-epicatechin. The method was suitable for assessing the plasma pharmacokinetics of (+)-catechin and (−)-epicatechin in rats after their individual oral administration as solutions in either water or milk.

Keywords: (+)-Catechin, (−)-Epicatechin, Oral absorption, Plasma, HPLC assay

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INTRODUCTION

(+)-Catechin (CAT) and (−)-epicatechin (ECAT) are two flavan-3-ol compounds representing the major monomeric flavonoid constituents present in unfermented cocoa beans,[1] cocoa powder,[2,3] and commercial chocolate.[2−5] Interest on these polyphenols stems from their ability to serve as antioxidants in biological and non-biological systems through a capacity to transfer electrons to free radicals, chelate transition metals, and activate antioxidant enzymes.[6,7] As a result, CAT and ECAT are endowed with potentially beneficial health related properties since free radicals such as superoxide anion, hydroxyl, and peroxyl are known to chemically alter cells,[6] lipoproteins,[6] and biomolecules,[6] and to be implicated in aging and in the development of atherosclerosis, cancer, inflammation, ischemic injury, and neurodegenerative disease.[6−8]

Studies in human subjects[1,9,10] and in rats[11−14] have shown than CAT and ECAT are rapidly absorbed from the gastrointestinal tract into the general circulation following their oral administration as solutions[11−14] or their ingestion as part of chocolate[9,10] or cocoa powder.[10] Furthermore, these studies have established the extensive biotransformation of CAT and ECAT to methylated, glucuronidated, and sulfated metabolites within the small intestine, liver, and kidney. While in humans, the ingestion of a single meal of chocolate has been found to yield measurable dose-related plasma levels of CAT and ECAT, which peak in 2−3 hours,[3,10] the oral administration of solutions of CAT and ECAT to rats led to plasma levels that peaked in 1−3 hours,[11] were higher for ECAT than for CAT,[11] and disappeared in about 6 hours.[11] In rats, the bioavailability of these flavonoids has been reported to be only about 5%.[14]

A report describing the influence of chocolate on the plasma antioxidant capacity of human subjects has suggested that the oral absorption of monomeric chocolate catechins decreases when the chocolate is consumed along with milk or it contains milk as an ingredient.[15] On the other hand, evidence of a lack of interaction between catechins in tea and chocolate with milk is also available.[16−18]

To more clearly demonstrate the effect that milk could have on the oral absorption of monomeric chocolate catechins, a study was undertaken in the rat in which CAT and ECAT were orally fed as solutions in water and in whole milk, and the ensuing plasma levels measured over time. In support of this study, the HPLC method described in the present report was developed, validated, and applied. This method can serve as an advantageous alternative to those proposed earlier for the same purpose and requiring a larger sample volume,[19,20] a more complicated and lengthier sample preparation step,[21,22] solvent gradient elution,[21,23] and/or detection with a photodiode array,[22,24,26,27] chemiluminescence,[25] or electrochemical[23,24] detector.

EXPERIMENTAL

Materials and Solvents

(+)-CAT hydrate (98%), (−)-ECAT, β-glucuronidase (3,000 U/mg, grade B3 from bovine liver), aryl sulfatase (10,000 U/g, grade H-1 from *Helix pomatia*), anhydrous sodium acetate, acetylsalicylic acid, formic acid, and EDTA disodium were obtained from Sigma Chemical Company, St. Louis, MO. Acetic acid (glacial) was from J.T. Baker, Phillipsburg, NJ, and perchloric acid (70%) was from Fluka Chemical Corporation, Milwaukee, WI. HPLC grade methanol and water were from EMD Chemical Inc., Gibbstown, NJ.

HPLC System

Chromatographic analyses were carried out on a liquid chromatograph consisting of Series 3B liquid chromatograph (Perkin-Elmer, Norwalk, CT), model RF-551 spectrofluorometric detector (Shimadzu, Riverwood Drive, MD), and model HP3396 Series II integrator (Hewlett-Packard, Avondale, PA). The samples were introduced through a model N6 injection valve fitted with a 50 μL sample loop (Valco, Houston, TX). The chromatographic separations were achieved on a Microsorb-MV C18, 25 cm × 4.9 mm i.d., 10 μm particle size, column.

Chromatographic Conditions

The mobile phase was a mixture of methanol-water-formic acid (15 : 84 : 1, by volume), filtered in vacuo through a 0.45 μm membrane filter (Millipore, Bedford, MA), and degassed by sonication prior to use. The mobile phase was pumped at a flow rate of 1 mL/min under isocratic conditions and ambient temperature. The detector settings were: excitation wavelength 280 nm, emission wavelength 310 nm, and attenuation 1 AUF. The integrator was set to operate at speed 0.1 cm/min, attenuation 2, threshold 2, and peak width 1.

Preparation of Standard Solutions

Stock solutions of a catechin compound (2.75 mg or its equivalent on an anhydrous basis) were prepared in methanol to a final volume of 250 mL.

Preparation of Internal Standard Solutions

(+)-Catechin hydrate (equivalent to 5.5 mg on an anhydrous basis) or (−)-epicatechin (5.5 mg) was placed in a 250 mL volumetric flask, dissolved

in methanol, and brought to volume with additional methanol. A working internal standard solution was prepared by mixing 3 mL of catechin compound stock solution with 2 mL of 70% perchloric acid. The mixture was stored in an amber glass container.

Preparation of Enzyme Solution

The enzyme solution was prepared by dissolving β-glucuronidase (6000 U) and aryl sulfatase (1650 U) in 5 mL of 0.1 M sodium acetate, previously adjusted to pH 5.0 with glacial acetic acid.

Validation of the Method

Linearity

Portions of stock solutions of CAT and ECAT in methanol, 22 μg/mL (160 nM) in concentration, were transferred to 2 mL stoppered polyethylene tubes so as to deliver 5, 10, 20, 40, 80, and 160 nM of catechin compound, and the solutions evaporated to dryness in a vacuum drying oven set to 37°C. Each residue was reconstituted in 100 μL of distilled water with the aid of sonication to yield an extract that was mixed and incubated at 37°C with 50 μL of enzyme preparation. After the addition of 50 μL of internal standard solution and vortex mixing, the reaction mixture was centrifuged at 6000 rpm for 10 min to remove precipitated proteins. An aliquot of the clear supernatant was injected into the liquid chromatograph. The same procedural steps were repeated with catechin extracts obtained by reconstituting the catechin residues with blank rat plasma rather than with distilled water. All samples were prepared in triplicate.

Recovery

The extraction efficiency was determined based on the peak area ratios obtained for the linearity study. In this case, the peak area ratios for solutions of a catechin compound added to rat blank plasma were compared with those derived from equipotent solutions in water. Assuming the detector response ratios for solutions in water to represent 100% recovery, the recovery of each catechin compound from plasma (as %) was calculated using the equation (detector response ratio of the catechin in plasma $\times$ 100)/(detector response ratio of the catechin in water).

Intra-day and Inter-day Precision

Intra-day reproducibility was evaluated by analyzing triplicate sets of blank rat plasma spiked with concentrations of CAT equal to 0.75, 3, and 12 nM

and of ECAT equal to 3, 6, and 12 nM, at three different times (0 hr, 4 hr, 8 hr). An assessment of interday method variability was based on the analysis of the same spiked plasma sample on days 1 and 3.

Stability Study

Plasma samples spiked either with CAT or ECAT (3 and 12 mM), and subjected to the deproteinization step, were stored at ambient temperature (25°C), 37°C and in a freezer (−20°C) for 4 days. During this time, each sample was analyzed by HPLC for intact catechin compound once daily.

Animal Studies

Groups of 6 male Sprague-Dawley rats, 275–300 g in weight, were fasted for 14 hr prior to the oral administration of a catechin compound. The catechin compounds were dissolved in either distilled water or in whole milk and delivered by the oral gavage technique, using a 14 gauge oral feeding needle and a 5 mL plastic syringe. In all instances the treatment dose was 350 mg/kg. Blood samples were collected from the tail vein every 0.5 hr for the first 2 hr, and at 1 hr intervals for the next 4 hr. About 0.5 mL of blood was collected each time in 2 mL stoppered polyethylene microtubes containing a small amount of disodium EDTA. After gentle mixing and centrifugation at 4000 rpm for 10 min, the plasma fraction was removed with the aid of a Pasteur pipette, transferred to a clean microtube, and stored at −20°C pending its HPLC analysis.

Sample Preparation

A frozen plasma sample from a rat previously dosed with an oral dose of a catechin compound was allowed to thaw at ambient temperature, and made uniform by gentle shaking. Then a 100 μL aliquot of plasma was transferred to a 2 mL stoppered polyethylene microtube, mixed with 50 μL of enzyme solution, and incubated at 37°C for 4 hr in an incubator. After allowing the mixture to cool to ambient temperature, it was treated with 50 μL of internal standard solution, vortex mixed for 30 sec, and centrifuged at 6000 rpm for 10 min. A portion of the clear supernatant was injected into the liquid chromatograph. Measurements were based on peak area ratios.

Data Analysis

Data for the method development study are reported as the mean ± SEM for $n = 6$ and the pharmacokinetic values are reported as the mean ± SEM for $n = 6$.

Pharmacokinetic parameters were calculated from the plasma CAT and ECAT levels using standard formulas and a computerized program (Microsoft Excel®, Microsoft Corporation, Redmond, WA), and the values analyzed for statistical differences by Student's t-test, one way analysis of variance (ANOVA), and Tukey's test for multiple comparisons using a commercial statistical software program (GraphPad Prism 4®, GraphPad Software, Inc., San Diego, CA). Differences were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

Sample Preparation and Analyte Stability

In comparison to sample preparation methods for the analysis of monomeric catechins in plasma and other biological samples that rely on the use of extraction into water-immiscible solvent,[23,26] solid-phase extraction,[26,28] extraction into an organic solvent followed by solid-phase adsorption-desorption,[2] deproteinization followed by solid phase adsorption-desorption,[23,24] or deproteinization followed by repeated extractions into a water-immiscible solvent,[20] the suggested sample preparation simply entails the addition of methanol-perchloric acid to the plasma sample.

Relative to deproteinization with organic solvents alone,[19,21] the present approach offers several advantages. For example, since maximal protein precipitation with any of these treatments is usually attained at a precipitant to plasma ratio >1.0, their addition to a biological sample will lead to a significant sample dilution and, hence, to a decrease in the amount of analyte available for detection. Although this procedural detail may not be as critical to fluorometric as is to photometric detection, it still merits consideration in situations where the availability of plasma sample is rather limited, as is the case during work with small laboratory animals. Thus, since in a typical experiment, 50 μL of methanol-perchloric acid (3 + 2) was very effective in deproteinizing a 100 μL volume of plasma sample, it will enable working with even smaller volumes of plasma as long as the ratio of plasma to deproteinizing agent remains 2 : 1. An additional advantage of this deproteinization approach is that it will remove plasma proteins as a dense and compact pellet that separates cleanly from the liquid phase. Methanol was used in preference to other organic solvents to make it compatible with the mobile phase. By itself, methanol was found to be a weak protein precipitant and, furthermore, to yield a protein precipitate that was neither compact nor adhering firmly to the bottom of the tube, even after extended centrifugation.

To ascertain the ability of methanol and of methanol in combination with either an acid (i.e., perchloric, phosphoric, trichloroacetic) or acetonitrile to remove plasma proteins, a study was conducted with these reagents and aliquots of catechin-free rat plasma. From the results of this study, which are graphically presented in Figure 1, it was determined that

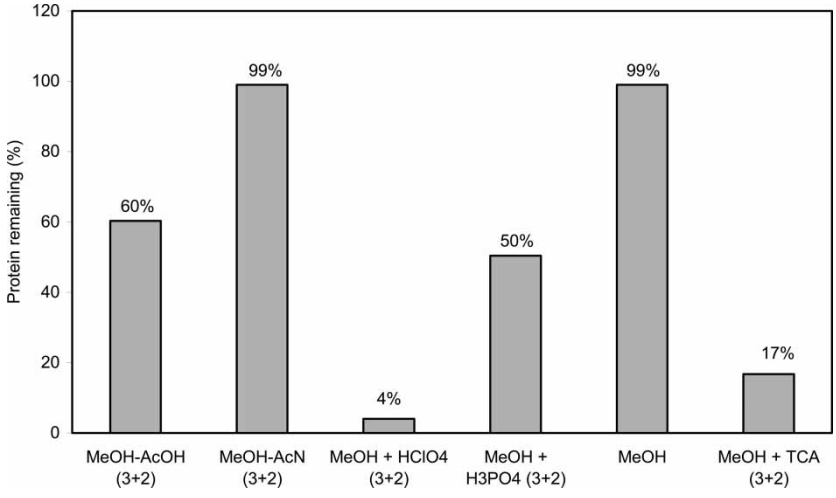

Figure 1. Amount of protein remaining after deproteinization of a rat plasma sample with six different deproteinizing solutions. The results represent the mean of duplicate samples.

methanol-perchloric acid (3 + 2) (96% removal) and, to lesser extent, methanol-trichloroacetic acid (2 + 1) (83% removal) were the most effective deproteinizing agents. In contrast, methanol and methanol-acetonitrile (1 : 1) removed only 1% of proteins; and replacing phosphoric acid for perchloric acid resulted in an almost 50% loss in efficiency relative to methanol-perchloric acid.

Assuming that the amount of catechin found in a solution in distilled water represented 100% recovery, the recoveries of CAT and ECAT from rat plasma spiked with 12 nM of either compound were much higher after treatment of the plasma sample with methanol-perchloric (3 + 2) (~99% for both catechins) than with methanol-trichloroacetic acid (3 + 2) (~41% for CAT, ~51% for ECAT) (Figure 2).

A separate study was conducted to investigate the effect that the perchloric acid in the deproteinizing solution could have on the chemical integrity of CAT and ECAT. For this purpose, aliquots of blank rat plasma samples were spiked with known amounts of CAT and ECAT treated with methanol-perchloric acid (3 + 2), centrifuged, and stored at 20°C, 25°C, and 37°C for up to 4 days. Daily HPLC analysis of these plasma samples for intact catechin revealed that the levels of CAT and ECAT remained quite stable during the storage at all temperature tested (data not shown). These results suggest the strong acidity created by the deproteinizing solution stabilized the flavan-3-ol structure, which is known to be susceptible to a temperature dependent epimerization[29] and degradation[22] in aqueous solutions of pH equal to or greater than 5.[22]

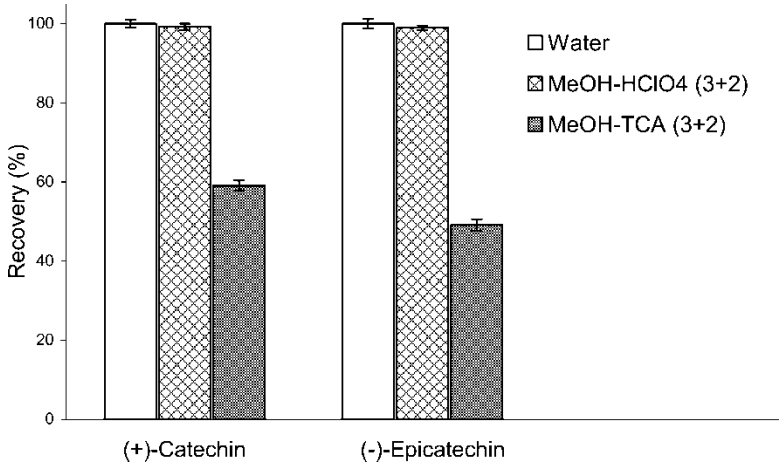

Figure 2. Comparison of the effects of methanol-perchloric acid (3 + 2) and methanol-trichloroacetic acid (3 + 2) on the recovery of (+)-catechin and (−)-epicatechin from aliquots of rat plasma spiked with 12 nM of each flavanol compound. Vertical bars represent the SD for triplicate samples.

Deconjugation Reaction

The circulating levels of CAT and ECAT that follow the oral absorption of these compounds in humans and laboratory animals are quite small, in part because of significant first-pass effect[30] and in part because of extensive metabolic conversion to sulfated, glucuronidated, and/or methylated forms in the small intestine,[11,12] liver,[12,13] and kidney.[13] The release of CAT and ECAT from their conjugated forms is readily accomplished by incubating the biological sample with a buffered mixture of β-glucuronidase and sulfatase to liberate the corresponding aglycones, which are then measured in terms of total CAT or ECAT levels. In the present study, the optimum incubation time for enzymatic hydrolysis was established by monitoring the changes in the peak height of the catechin of interest over time, which was found to level off after 4 hours. The enzymatic potency of the deconjugating mixture was adjusted to represent an excess over a potency reported in the scientific literature.[13]

Chromatographic Conditions

A fluorometric detector was used to monitor the elution of the catechin compounds, in preference to a photometric or electrochemical detector, taking into account the results of earlier studies that found fluorometry to be more sensitive for the detection of CAT and ECAT in plasma samples than either photometry[19,24] or electrochemistry.[24]

By examining the effect of the ratio of water to methanol in the mobile phase on the elution times of CAT and ECAT, a ratio effecting baseline separation between the peaks of interest and between these peaks and those of endogenous plasma components (Figures 3A and 3B) was established. Furthermore, to correct for the inability of aqueous methanol to produce narrow and symmetrical peaks, a small amount of an acid, such as formic acid was also included in the final mobile phase. With this mobile phase and a flow rate of 1 mL/min, the analysis of a single monomeric catechin

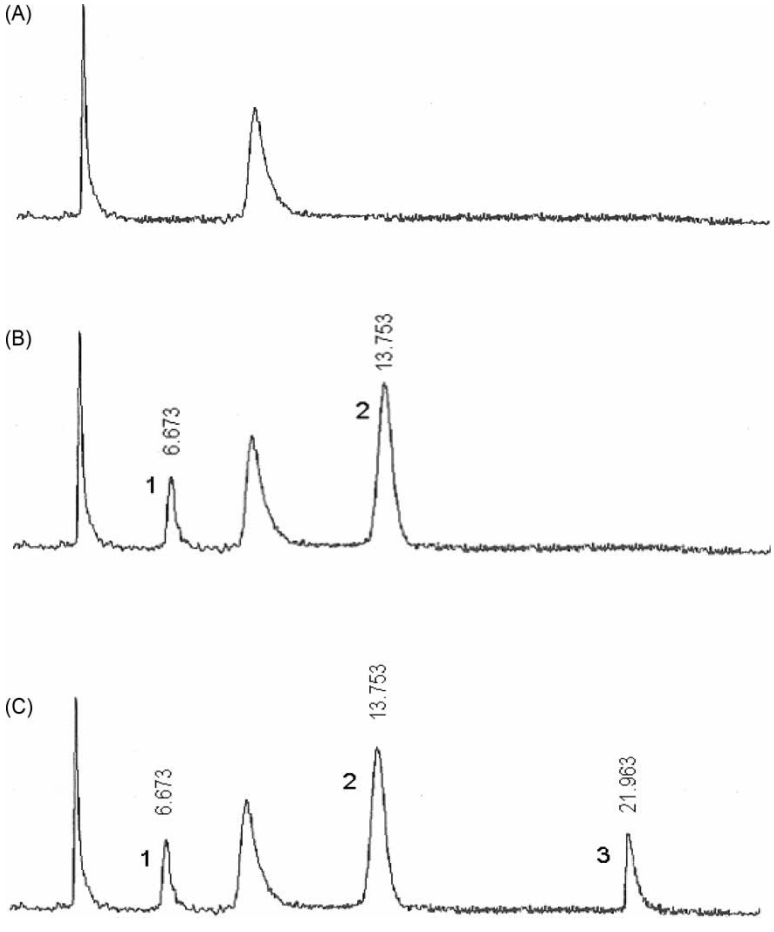

Figure 3. Typical chromatograms of: (A) a catechin-free rat plasma, (B) a catechin-free rat plasma spiked with 1, (+)-catechin and 2, (−)-epicatechin, and (C) a catechin-free rat plasma spiked with 1, (+)-catechin, 2, (−)-epicatechin, and 3, acetylsalicylic acid, the internal standard.

could be completed in less than 18 min. Also, since CAT (retention time = 6.7 min) and ECAT (retention time = 13.8 min) were well resolved from each other (resolution factor = 25.2), and since they were going to be administered individually to rats *in vivo* pharmacokinetic studies, it was possible to use one of them as the internal standard of the other. However, in the event that CAT and ECAT are going to be analyzed together, acetylsalicylic acid (retention time 21.9 min, Figure 3C) will serve as an appropriate internal standard.

Linearity and Limits of Detection

The linearity of the proposed HPLC method was studied by preparing six-point calibration curves of CAT and ECAT, both in water and in blank rat plasma. For both types of solution, peak area ratios were found to be directly proportional to concentrations of CAT and ECAT in the range 1.5–12 nM. The line equations for each of these catechins are listed in Table 1. Regression coefficient values in water and in plasma were respectively 0.9931 and 0.9957 for CAT and 0.9972 and 0.9985 for ECAT. The lowest concentrations of CAT and ECAT that could be accurately measured by the proposed method at a signal to noise ratio of 2 : 1 were 0.75 nM and 1.5 nM, respectively.

Precision and Accuracy

The intra-day variability of the proposed HPLC method was assessed by measuring triplicate samples of CAT or ECAT in blank plasma, to represent concentrations of CAT in the range 0.75–12 nM and of ECAT in the range 3–12 nM, at three different times within the same day. The RSD values ranged from ~1.8–2.1% for CAT and from 0.04–2.9% for ECAT (Table 2). The RSD for interday assays of the same plasma samples conducted on days 1 and 3 were in the range 4.1–1.3% for CAT and

Table 1. Line equations derived from the linearity study of CAT and ECAT in water and rat plasma[a,b]

	Water		Plasma	
Compound	Line equation	r^2	Line equation	r^2
CAT	$y = 0.1025x + 0.0022$	0.9931	$y = 0.0989x - 0.0365$	0.9957
ECAT	$y = 0.2519x - 0.0373$	0.9972	$y = 0.2669x - 0.0921$	0.9985

[a]Concentration range studied: 5–160 nM.
[b]r^2 = Regression coefficient based on the best line for triplicate samples.

Table 2. Results of the intraday variability study on the proposed HPLC method based on three different concentrations of CAT and ECAT added to blank rat plasma[a,b]

Concentration (nM)	Peak area ratio (mean ± SD, n = 4)	RSD (%)
CAT		
0.75	0.129 ± 0.002	1.78
3.00	0.283 ± 0.004	1.27
12.00	1.222 ± 0.026	2.09
ECAT		
3.00	0.763 ± 0.001	0.04
6.00	1.501 ± 0.001	0.13
12.00	3.176 ± 0.091	2.88

[a]Assays were carried out at 0, 2, 4 and 6 hr.

~2.0–0.5% for ECAT, respectively (Table 3). To determine the extent of recovery from plasma samples, blank rat plasma was spiked with amounts of CAT and ECAT in the concentration ranges 0.75–12 nM and 1.50–12 nM, respectively. Recoveries for CAT ranged from 95–101.7% and for ECAT from 98.7–102.5% (Table 4)

Plasma Pharmacokinetic Studies

The utility of the proposed HPLC method in assessing the oral pharmacokinetics of monomeric catechins was investigated in rats. For this purpose,

Table 3. Results of the interday variability study on the proposed HPLC method based on three different concentrations of CAT and ECAT added to blank rat plasma[a,b]

Concentration (nM)	Peak area ratio (mean ± SD, n = 4)	RSD (%)
CAT		
0.75	0.124 ± 0.005	4.07
3.00	0.278 ± 0.009	3.15
12.00	1.248 ± 0.016	1.27
ECAT		
3.00	0.786 ± 0.016	2.04
6.00	1.562 ± 0.013	0.80
12.00	3.012 ± 0.014	0.47

[a]Assays were carried out on days 1 and 3 of the study.

Table 4. Results of recovery of CAT and ECAT from spiked rat plasma

Amount added (nM)	Amount found (mean ± SD, n = 3)			
	CAT (nM)	ECAT (nM)	CAT (%)	ECAT (%)
0.75	0.75 ± 0.01	ND[a]	100.00 ± 1.00	ND
1.50	1.47 ±0.01	1.48 ± 0.01	98.00 ± 1.20	98.70 ± 1.10
3.00	2.85 ±0.01	3.00 ± 0.01	95.00 ± 0.99	100.00 ± 1.10
6.00	6.10 ±0.02	6.13 ± 0.02	101.67 ± 2.11	102.17 ± 2.15
12.00	12.19 ±0.01	12.30 ± 0.01	101.33 ± 1.12	102.50 ± 2.50

[a]ND = not done.

solutions of CAT or ECAT, either in distilled water or whole milk, were orally fed to groups of six rats each by the gavage technique, at a dose of 350 mg/kg. Thereafter, blood samples were collected periodically from the tail vein for up to 5 hr post administration. The assay of the corresponding plasma fractions yielded the plasma concentration versus time profiles shown in Figure 4. The plasma concentrations of CAT and ECAT were used to calculate the pharmacokinetic values presented in Table 5, which were fitted to a non-compartment model. Regardless of the vehicle used, the changes in plasma concentration curves for the two solutions of CAT did not differ markedly from each other in terms of the pattern of rise and t_{max} characteristics

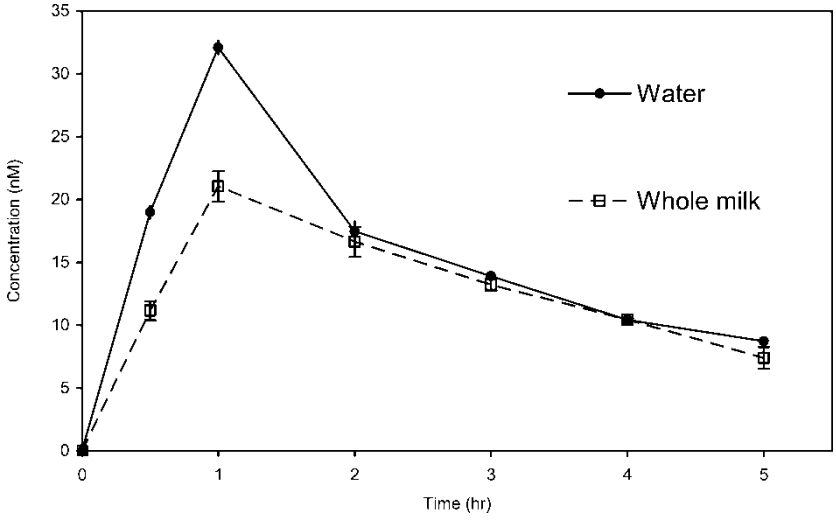

Figure 4. Changes in mean plasma concentration over time of (+)-catechin following its oral dosing as a single 350 mg/kg dose as a solution in water and whole milk to rats. Vertical bars represent the SEM for 6 rats.

Table 5. Plasma pharmacokinetic values (mean ± SEM, n = 6) for oral doses of CAT and ECAT (350 mg/kg each) administered to rats as aqueous solutions and as solutions in whole milk[a]

	Water		Whole milk	
Parameter	CAT	ECAT	CAT	ECAT
AUC ($mM \cdot hr^{-1}$)	79.74 ± 0.29	59.17 ± 0.35	65.36 ± 0.63	54.29 ± 0.33
C_{max} (mM)	32.09 ± 0.56	25.08 ± 0.55	21.04 ± 1.21	19.39 ± 0.64
t_{max} (hr)	1.00 ± 0.00	1.00 ± 0.05	1.00 ± 0.00	3.00 ± 0.15
$t_{1/2}$ (hr)	2.22 ± 0.11	1.18 ± 0.06	2.71 ± 0.14	2.13 ± 0.11
k_a (hr^{-1})	32.09 ± 0.31	25.08 ± 0.36	21.04 ± 0.59	6.29 ± 0.05
k_e (hr^{-1})	0.31 ± 0.02	0.59 ± 0.03	0.26 ± 0.01	0.33 ± 0.02

[a]For each flavanol compound, differences between water and whole milk were statistically significant at $p < 0.001$ by Student's t-test and one-way ANOVA except for the t_{max} values.

(Figure 5). However, relative to a solution in water, milk lowered the AUC (by 12%), C_{max} (by 34%), k_a (by 34%), and k_e (by 18%) values, and lengthened the $t_{1/2}$ (by 22%) of CAT. Even though the rise and fall of the plasma levels of ECAT over time did not differ markedly from those of CAT when these compounds were delivered as aqueous solutions, some important

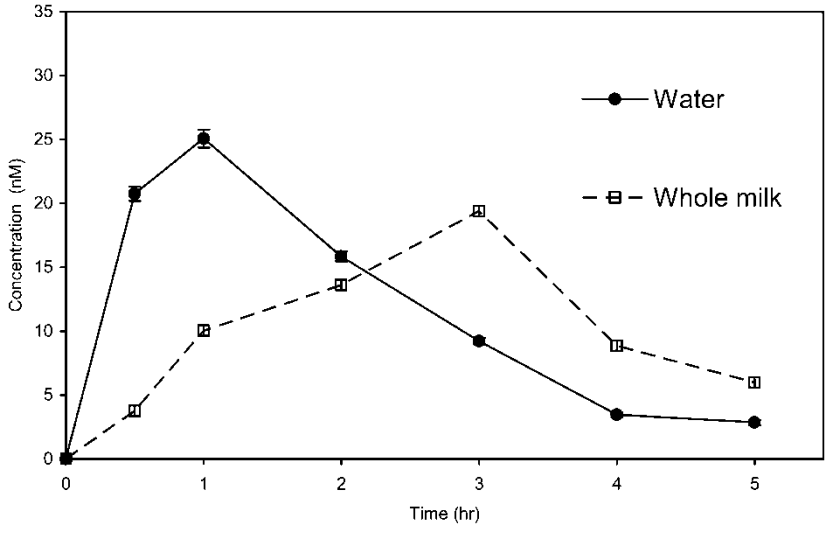

Figure 5. Changes in mean plasma concentrations over time of (−)-epicatechin following its oral dosing as a single 350 mg/kg dose as a solution in water and whole milk to rats. Vertical bars represent the SEM for 6 rats.

differences could be established between them. Thus, the AUC, C_{max}, and k_a values for ECAT were lower (by 26%, 22%, and 22%, respectively), the $t_{1/2}$ was longer (by ~1 hr), and the k_e value higher (by 88%) than those of CAT. On the other hand, the administration of ECAT as a milk solution resulted in lower AUC, C_{max}, k_a, and k_e values (by 8%, 23%, 75%, and 45%, respectively), a delayed t_{max} (by ~2 hr), and a longer $t_{1/2}$ (~0.99 hr) relative to an equipotent solution in plain water. In general, whole milk was found to impair the oral absorption and ensuing plasma pharmacokinetics of both CAT and ECAT, with the effect being much greater on ECAT than on CAT.

CONCLUSIONS

The present HPLC method is shown to be simple, rapid, accurate, reproducible, and possessing good linearity and sensitivity. The simplicity of the sample preparation step makes the method suitable for the analysis of the large number of samples that is generated in a typical pharmacokinetics study. Furthermore, by maintaining the suggested ratio of deproteinizing solution to plasma sample, the volumes of reagents required for sample preparation can be readily adjusted to the volume of plasma available, and the limits of detection preserved. This flexibility may be advantageous in the course of pharmacokinetic studies with small laboratory animals and where the sample size is often limited.

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