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# Investigating the Stability of EGCg in Aqueous Media

*(-)-Epigallocatechin gallate (EGCg) is the most prevalent catechin in green tea extract, to which most of the health benefit of green tea has been attributed. Since EGCg is an antioxidant, its stability in various biological fluids must be evaluated prior to the study of its* in vivo *pharmacokinetics and pharmacodynamics. For this purpose, a multi-channel LC/EC method was developed to determine EGCg quantity at a concentration very likely to be found* in vivo *(<500 ng/mL). A microbore column was used to minimize sample consumption. The detection limit for EGCg was 0.8 ng/mL at a potential of +600 mV vs. Ag/AgCl. The calibration curve was linear over the range of 1-500 ng/mL. Using this method, the stability of EGCg (100 ng/mL) in 10 mM HCl, saline and Ringers' solution, with or without preservatives, was monitored. It was found that EGCg was very stable in all these solutions at low temterature only when they were free of certain metal ion contaminants. Therefore, it is suggested to stabilize EGCg solutions by use of a metal scavenger (EDTA), an antioxidant (e.g. ascorbic acid), keeping the pH below or close to neutral and keeping the temperature cold during sampling and storage of EGCg.*

Tea, the dried leaf of *Camillia sinensis*, has been widely consumed as a beverage for more than 4000 years, and its medicinal applications have been actively studied with scientific methods in this decade. Most of its biological activities such as lipid-lowering (1,2), antimicrobial (3), anti-obesity (4-6), anticancer and antimutagenisis (7-9), are found to be related to its polyphenol constituents, especially the tea catechins. Much effort has been devoted to assess the bioavailability and pharmacokinetics of tea catechins (10-15). Since all catechins are potent antioxidants, they tend to be oxidized easily within the biological environment. Therefore, the stability of tea catechins must be evaluated so reliable results can be obtained from any *in vivo* pharmacokinetic or pharmacodynamic studies. Only limited reports are found regarding this area (16-18). Since (-)-epigallocatechin gallate (EGCg, ***F1***) is the most prevalent and interesting catechin in green tea extract, its stability in various media was examined in this study.

The compound was quantitated by an LC/EC method at a concentration likely to be found *in vivo*. Liquid chromatography/electrochemical detection (LC/EC) has long been proven to be a very sensitive and selective method analyzing the antioxidant components in complex mixtures (19-24). In this study, a C18 microbore column was used to reduce the sample amount required for the quantitative analysis, a prerequisite which might be important for future *in vivo* studies of EGCg where only limited samples can be collected from animals, as *in vivo* microdialysis or multiple blood sampling from small animals.

## Experimental

### Apparatus

The LC/EC system consisted of a chromatographic pump (PM-80, BASi, West Lafayette, IN, USA), a C18 microbore column (100 x 1 mm, 5 µM, BASi, MF-8949), and a multi-channel amperometric detector (epsilon™ BASi) coupled to four glassy carbon working electrodes in an arc flow thin-layer cell. Potentials of +600, 500, 450, 400 mV vs. Ag/AgCl were applied to the working electrodes. The mobile phase contained 0.6 % (v/v) formic acid and 8% acetonitrile in water, which was delivered at 0.15 mL/min. All samples were injected via a 5.3 µl loop by a refrigerated autosampler (CMA, Sweden). Data were acquired and integrated with BASi ChromGraph® software.

### Chemicals and Reagents

EGCg and vitamin C were purchased from Sigma (St. Louis, MO, USA). Analytical grade $CaCl_2$, KCl, NaCl, $NaH_2PO_4$, ethylenedinitrilo tetraacetic acid disodium salt (EDTA) and 88% formic acid were purchased from Mallinckrodt (Phillipsburg, NJ, USA). $CuCl_2$ (1000 ppm standard solution in water) was purchased from Ricca Chemicals Co. (Arlington, TX, USA). $FeBr_2$ and $FeCl_3$ were purchased from Aldrich (Milwaukee, WI, USA). Saline solution in bags (for injection) and in bottles (for irrigation) were obtained from Abbott (Chicago, IL, USA). The Ringers' solution was purchased from B. Braun Medical Inc. (Irvine, CA, USA). Acetonitrile was of HPLC grade (Burdick and Jackson, Muskegon, MI, USA). Reagent grade water was

prepared from in-house deionized water using a NANOpure system (Barnstead/ Thermolyne, Dubuque, IA, USA).

The EGCg stock solution was prepared in 10 mM HCl at a concentration of 1.0 mg/mL, and was kept at -20°C in the dark until used. It was diluted to 100 ng/mL (i.e., 0.2 µM) in various media as desired. All samples were stored at -20°C in the dark. A calibration curve was constructed by diluting the stock solution in bottled saline solution to yield the final concentrations of 1, 2, 5, 50 and 500 ng/mL.

The Vc preservative solution contained 2% ascorbic acid and 0.05% EDTA in 0.4 M $NaH_2PO_4$ solution, which was kept at -4°C until used. Stock solutions of EDTA at 1 mM, Fe(II) and Fe(III) at 1 ppm were prepared by dissolving the appropriate amount of salts (EDTA, $FeBr_2$ and $FeCl_3$, respectively) in water. Homemade Ringers' solution was prepared by dissolving the appropriate amount of $CaCl_2$, KCl and NaCl in water.

## Results

In this study, a sensitive multi-channel LC/EC method was developed to determine EGCg quantity at a concentration very likely to be found *in vivo* (<500 ng/mL). For the first time, the analysis was acomplished with a C18 microbore column, which could further reduce the amount of sample required for the quantitative analysis. This offers great potential for future *in vivo* study of EGCg where only limited samples can be collected from animals. The detection limit for EGCg was 4.3 pg on column at a potential of +600 mV vs. Ag/AgCl reference electrode. The calibration curve was linear over the range of 1-500 ng/mL (***F2***).

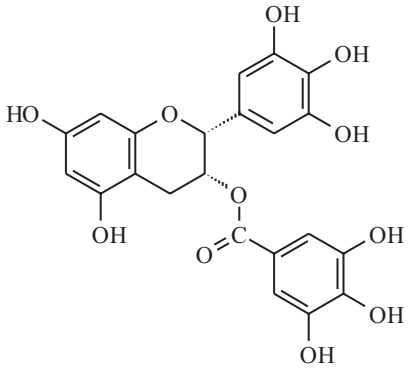
When this method was applied to the stability study of EGCg in various aqueous solutions, it was found that the stock solution in 10 mM HCl was very stable at -20°C for at least a week (***F3***). On the other hand, the EGCg dissolved in the Ringers' or saline solution (both were commercially available bagged solutions in neutral pH) registered a significant loss at 25% to 90%, respectively, by day three or day four (***F3***). Similar results were obtained when bagged saline from a different lot was used. At first, it was concluded that EGCg at low concentration was only stable in acidic media. When it was tested in another type of saline solution that was also commercially available but came in bottles (for irrigation) however, EGCg demonstrated excellent stability over the same period (***F4***). In addition, EGCg appeared to be very stable in homemade Ringers' solution which had exactly the same composition as the purchased bagged solution (***F4***). Such results implied that the degradation observed in the previous experiments was not due to the neutral pH or to any major salts presented in the Ringers' or saline solutions.

In the previous experiments, bagged Ringers' or saline solutions were pulled through a syringe needle before being used to dilute the EGCg stock solution. Therefore, it was suspected that trace amounts of metal ions might have been introduced into the saline or Ringers' solutions, which could catalyze the degradation of EGCg. When Fe(II), Fe(III), and Cu(II) at 2 ppb (36, 31 nM, respectively) were added to the EGCg solution in bottled saline, dramatic degradation of EGCg was observed in those solutions containing Cu(II) ions (***F5***). Furthermore, such degradation could be reduced by the addition of EDTA (1 µM) in excess (***F5***). It was not clear how the Cu(II) ions facilitated EGCg degradation, although a catalytic role rather than an oxidative role was suspected. It was observed that such degradation was not reversible by the later addition of EDTA to the solution.

Since in most *in vivo* sampling methods, metal parts (needles, connector, etc.) would inevitably be involved, it is important to find ways to protect EGCg against possible contamination by metal ions such as Cu(II). When a relatively large amount of EDTA (65 µM) was added to bagged saline before it was used to dilute the EGCg stock solution, it preserved EGCg to different degrees in different samples (***F6***). When 5% (v/v) Vc preservative soution was added to the bagged saline or Ringers' solution, most EGCg was preserved for three days (***F6***). The final concentrations of ascorbic acid and EDTA in these solutions were 5.6 mM and 65 µM, respectively. Therefore, the degradation of EGCg observed in the first series of experiments may be due not only to contamination of metal ions, but also to some other unknown oxidizing agents in bagged solutions pulled through the syringe needles. It is best to add ascorbic acid as a scavenger in addition to EDTA to preserve the EGCg in any biological samples.

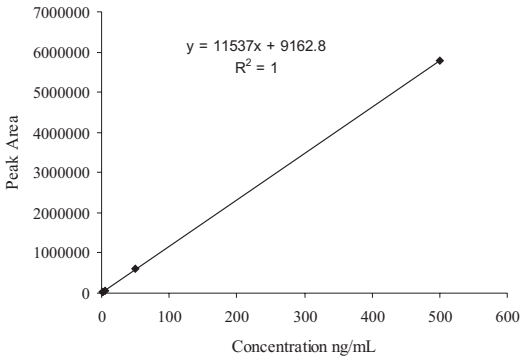
**F1.**

(-)-Epigallocatechin gallate




**F2.**

Calibration curve of EGCg in saline. Amperometric detector: +600 mV vs. Ag/AgCl.




## Conclusion

Since EGCg is a potent antioxidant, easily oxidized in biological media, its stability must be evaluated prior to any

*in vivo* pharmacokinetic or pharmacodynamic study. A multi-channel LC/EC method was employed to determine EGCg at a concentration most likely to be found *in vivo* (100 ng/mL). It was found that EGCg was very stable in saline or the Ringers' solutions at low temperature, unless there were certain metal ion contaminants present. Therefore, there are clear advantages to stabilizing EGCg solutions by using a metal scavenger (EDTA), an antioxidant (e.g., ascorbic acid), keeping the pH somewhat below neutral and keeping the temperature low during sampling and storage of EGCg.

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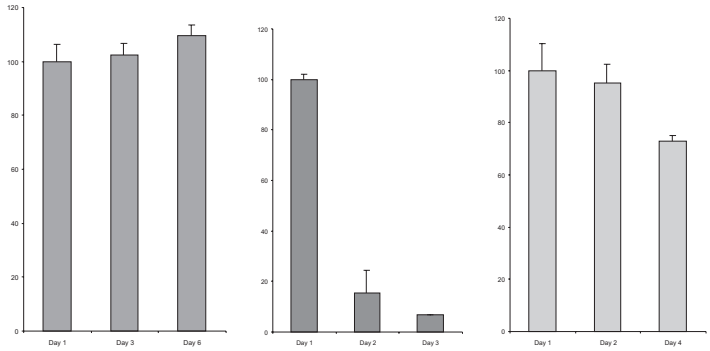
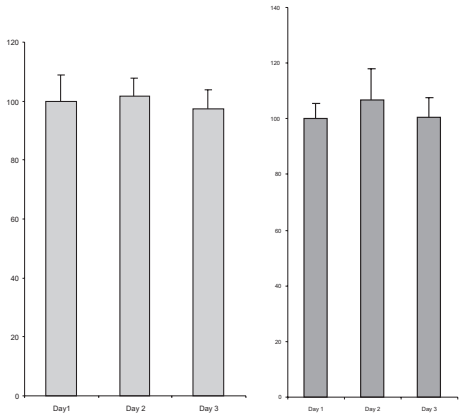
**F3.**
Stability of EGCg at 100 ng/mL in (A) 10 mM HCl; (B) bagged saline; (C) bagged Ringers' solution.




A B C

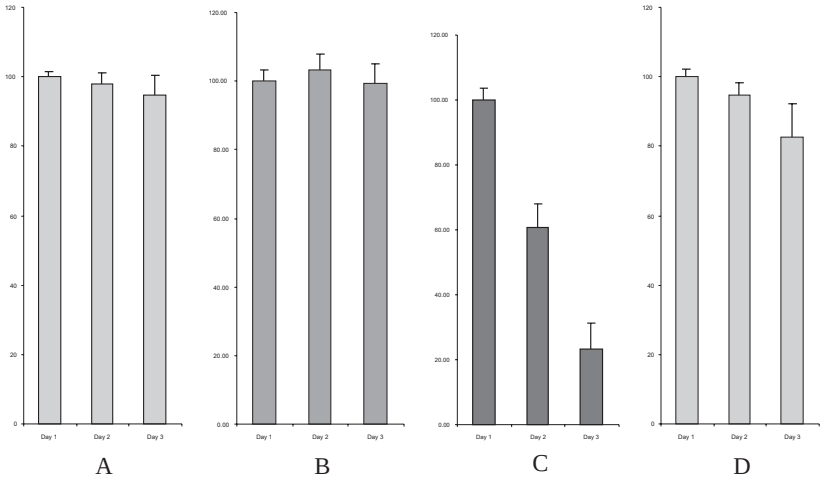
**F4.**

Stability of EGCg at 100 ng/mL in (A) bottled saline; (B) homemade Ringers' solution.




**F5.**

Stability of EGCg at 100 ng/mL in bottled saline containing (A) Fe(II) at 36 nM; (B) Fe(III) at 36 nM; (C) Cu(II) at 31 nM; (D) Cu(II) at 31 nM and EDTA at 1 µM.




**F6.**

Stability of EGCg at 100 ng/mL in (A) bagged saline containing EDTA at 65 µM; (B) bagged saline containing EDTA at 65 µM and vitamin C at 5.6 mM; (C) bagged Ringers' solution containing EDTA at 65 µM and vitamin C at 5.6 mM.