Printed in the Republic of Korea
ANALYTICAL SCIENCE & TECHNOLOGY
Vol. 28 No. 2, 112-116, 2015
http://dx.doi.org/10.5806/AST.2015.28.2.112

Simultaneous determination of betaine and choline using derivatization by HPLC with UV detection

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(Received March 2, 2015; Revised April 8, 2015; Accepted April 8, 2015)

HPLC-UV검출방법으로 유도체화를 통한 비테인과 콜린의 동시분석

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Abstract: Extraction of quaternary ammonium compounds (choline and betaine) from plant samples (spinach) using ion exchange resin (AG1, OH^- form) is a very simple and inexpensive approach. However, it is very hard to determine amounts of choline and betaine simultaneously using high-performance liquid chromatography-ultraviolet (HPLC-UV) detection. Unlike choline, betaine has low molar absorptivity in UV-visible (UV-Vis) region, which makes it difficult to carry out UV-Vis detection of betaine. The mixture of quaternary ammonium compounds (choline and betaine) was derivatized using 2-bromo acetophenone as a derivatizing agent. As a result, choline did not react with the derivatizing agent, whereas betaine formed a betaine derivative. This betaine derivative exhibited detectable UV absorption with baseline separation between choline and the betaine derivative. Thus, with this method, choline and betaine can be determined simultaneously by using the HPLC-UV method through one-step derivatization, which is an easy, sensitive, and reliable method.

Key words: choline, betaine, derivatization, HPLC, UV detection

1. Introduction

Many plant species accumulate the osmo-protectant glycine betaine in response to salt or drought stress.[1-2] Choline is a major constituent of the lipid fraction in biological membranes and free choline is frequently present in plants and animals.[3] Betaine is a major choline metabolite in liver and kidney, and may be important product of choline metabolism in other tissues.[4] Glycine betaine and its precursor choline have been found to confer a high level of osmotic tolerance when added exogenously to cultures of Escherichia coli at an inhibitory osmotic strength.[5] Glycine betaine is a dipolar ion possessing a quaternary ammonium group and a carboxylic acid group.[6] The quaternary ammonium compounds, choline and betaine, have a

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variety of effects and are closely connected metabolically.[7-8]

Various methods have been reported for the measurement of plasma free choline, including radioenzymatic assays,[9-10] gas chromatography,[11-12] HPLC with electrochemical detection[13,14] and HPLC-mass spectrometry (MS).[15] Also many methods are applied for the assay of betaine in the literature.[16-18] Extraction of quaternary ammonium compounds (choline and betaine) from plant sample (spinach) using ion exchange resin (AG1, OH^- form) is very simple and inexpensive method. While the sensitive methods for assaying choline are available, assay of betaine has been known as difficulty.

Dawson *et al*.[19] has reported about the Dragendorff reagent, which was used for detection lacked sensitivity, with the colorimetric reaction being only semi-quantitative. Various techniques are also available for this purpose, viz. pyrolysis gas chromatography,[20] HPLC,[21-23] spectrometric methods[24-26] etc. However the techniques are expensive and require sophisticated analytical methods. Sometimes, the sample preparation methods are quite complicated. Mar *et al*.[27] reported a single HPLC column and solvent system for the separation of betaine from other choline metabolites using 4-bromophenacyl triflate as a derivatizing agent. That derivatization was prepared from 4-bromo-2 diazoacetophenon which generates diazomethane during the reaction. Due to its high toxic and explosive nature, they had to take proper safety precautions.

Here, we present a method which is a comparatively very simple and no need to take such kind of safety precautions. The mixture of choline and betaine was derivatized using 2-bromo acetophenone as a deriva- tizing agent. In this way, only betaine forms derivative through one-step derivatization, and thus choline and betaine can be determined simultaneously by using HPLC-UV method which is easy, sensitive and reliable.

2. Experimental

2.1. Reagents

The standard choline chloride and glycine betaine were used as reagent grade. 2-bromo acetophenone was used as a derivatizing reagent. All chemicals were purchased from Aldrich (Milwaukee, USA). Deionized water was prepared by passing in house distilled water through a Human power water system (Human Corporation, Korea). All reagents and solvents were of analytical grade and were used without further purification.

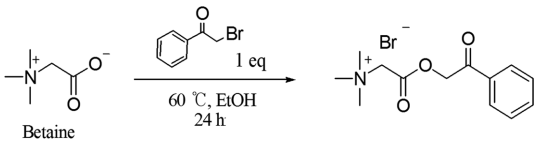

Fig. 1. The derivatization of betaine.

2.2. Derivatization of betaine

For the synthesis of betaine-derivative, the same equivalent of betaine (0.59 g, 5.02 mmol) and 2-bromoacetophenone (1.00 g, 5.02 mmol) was mixed in ethanol at 60°C for 24 h.[28] TLC analysis has been done using UV lamp (254 nm) and indicates the formation of betaine-derivative. The reaction mixture was quenched at room temperature and worked up using methylene chloride. Finally it was washed with $Na_2S_2O_3$ and water. The mixture was dried by $MgSO_4$ and isolated by column chromatography. The final product of betaine-derivative synthesis was confirmed with NMR. The reaction scheme for the derivatization of betaine is given in *Fig.* 1.

2.3. Sample preparation

In the process of extraction of osmo-protectants, the spinach samples were freeze-dried, kept in a desiccator, and finally ground just before extraction of choline and betaine from the plant sample.[29] Then, the samples (200 mg) were mixed with 5 mL of a mixture of ethanol/water (9:1, v/v). The AG1 resin (200-400 mesh, OH^- form, Bio-Rad) was used in a small column (4.0 mL) and dried down by centrifugation before being loaded with 250 μL of crude extract. The column was then washed with 750 μL of distilled water. The whole effluent was collected by another centrifugation.

2.4. Preparation of standard solution

Standard stock solution of 100 μg mL^{-1} choline chloride and betaine-derivative were prepared by weighing accurately about 100 mg of choline chloride and betaine-derivative with distilled water into a 1000 mL volumetric flask. The working standard solutions of 5, 10, 20 and 50 μg mL^{-1} choline chloride and betaine-derivative were made by appropriate dilution of the stock solution with distilled water.

2.5. High-performance liquid chromatography system

The HPLC system is consisted of a solvent delivery pump (model 2800, BIO RAD), an injector (model 7125i, Rheodyne) with a 20 μL sample loop, and a photo diode array detector series (model 1050, Hewlett Packard). A silica column (250 mm × 4.6 mm, 80 A, 4 μL) was purchased from Phenomenex (Torrance, CA, USA).

2.6. Chromatographic conditions

A silica column was used for separation. Mobile phase was prepared by methanol : water (4:1, v/v). It was filtered using a 0.45 μm filter and degassed for several minutes prior to use. The separation was performed at a mobile phase flow-rate of 0.8 mL min^{-1}. The column temperature was ambient. The injection volume for samples with internal standard (2,4-dichlorophenol) was 25 μL.

3. Results and Discussion

3.1. Choice of maximum UV absorbance

The maximum UV absorbance of choline and betaine were occurred at the same wavelength of 200 nm. Betaine, unlike choline, has low molar-absorptivity and it makes difficulty to detect betaine with UV detection. Thus choline and betaine were derivatized with using 2-bromo acetophenone as a derivatizing agent. From those, choline does not react with the derivatizing agent. However betaine forms betaine-derivative which shows the maximum UV absorbance at 254 nm with high molar absorptivity.

The chromatogram of choline chloride standard and betaine standard (10 mg L^{-1}, each) at 200 nm is shown in *Fig.* 2(A). Two peaks of choline chloride and betaine appears completely overlap at 6.5 min

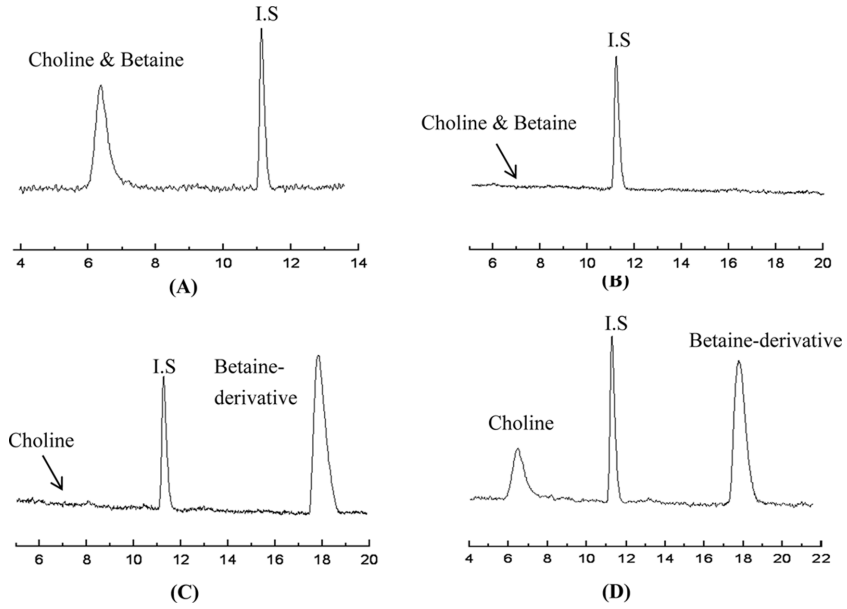

Fig. 2. Chromatograms of (A) mixture of choline chloride and betaine at 200 nm, (B) choline chloride and betaine at 254 nm, (C) choline chloride and betaine after derivatization at 254 nm (D) choline chloride and betaine-derivative at 230 nm.

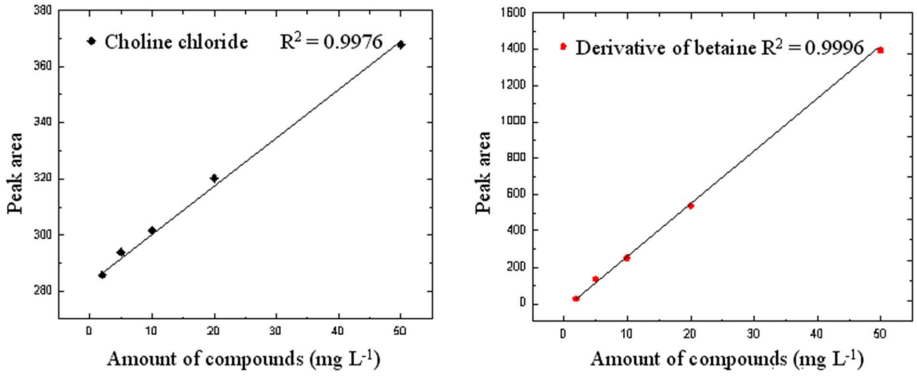

Fig. 3. Calibration lines of choline chloride and betaine-derivative (n = 5).

after injection.

The chromatograms of choline chloride standard and betaine standard (10 mg L^{-1}, each) at 254 nm are given in *Fig.* 2(B) and *Fig.* 2(C) as before and after derivatization, respectively. The peaks for standard choline chloride and betaine and betaine-derivative have been indicated. From the results obtained, we decided to use 230 nm as suitable detection wavelength to determine both of analytes simultaneously. *Fig.* 2(D) represents the chromatogram of standard choline chloride and betaine-derivative at 230 nm and the clearly distinguishable peaks of choline chloride and betaine-derivative are observed in the chromatogram.

3.2. Calibration curves

Calibration curves for choline chloride standard and betaine-derivative were constructed by varying the amounts of each analytes to their relative response factors as determined by the ratio of the peak area of analytes (*Fig.* 3). Responses for all the analytes show good linearity and the correlation coefficients (R^2) values of choline and betaine-derivative were 0.9976 and 0.9996, respectively.

Based on the optimized conditions of sample preparation, we obtained relative recovery data (80.5-93.4%) from the different concentrations (20.0 mg L^{-1} for choline and 10.0 mg L^{-1} for betaine). Reproducibility was obtained as 1.5~6.0% RSD, which was affected by the applied concentration.

3.3. HPLC-UV analysis of plant samples

The newly developed method was applied to real sample study. Spinach was chosen and through extraction conditions, choline and betaine extracted from spinach were confirmed by HPLC-UV at 230 nm. *Fig.* 4(A) represents the chromatogram of choline and betaine after extraction from spinach and it

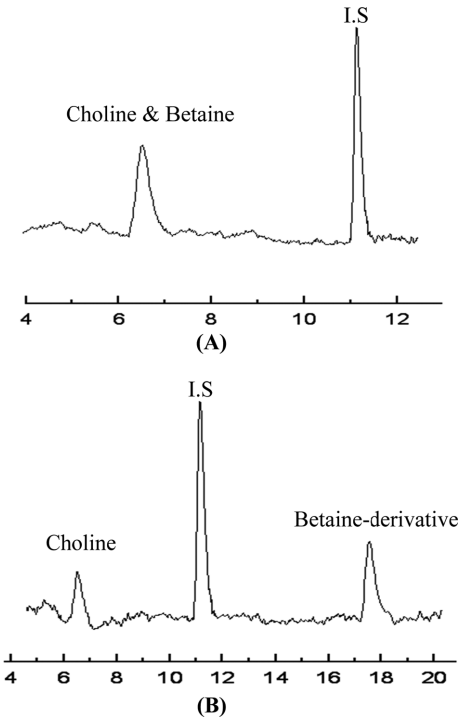

Fig. 4. Chromatograms of betaine and choline in the extract from real plant sample at 230 nm (A) without derivatization and (B) with derivatization.

shows single peak. However, the chromatogram of *Fig.* 4(B) was obtained with extraction and derivatization from spinach and it shows of two distinct peaks of choline and betaine-derivative.

4. Conclusions

Aqueous extract of plant sample (spinach) over ion exchange resin (AG1, OH^- form) gives choline and betaine in the collected effluent. This procedure is very easy and economic one. Choline and betaine cannot determine simultaneously before derivatization as it is seen in the chromatogram. However, simultaneous determination of choline and betaine is possible after derivatization using 2-bromo acetophenone as a derivatizing agent.

The main topic of this note is to develop a novel sensitive method for the simultaneous determination of choline and betaine with HPLC-UV after one-step derivatization, which is relatively easy and comparatively cheap.

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