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An improved method for the determination of betaine in *Echinacea* products

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A rapid and sensitive HPLC method for the separation and quantification of betaine in *Echinacea* products has been developed. Strong cation-exchange (SCX) material was used as stationary phase, and a mixture of methanol and 50 mM choline buffer (pH 3.5) as mobile phase. After formation of the bromophenacyl derivative, betaine was detected at 254 nm with a detection limit of 0.2 μg/ml. The method was successfully used to analyze several *Echinacea* market products, and significant variations in their betaine content from 0.04 to 0.64% were observed.

1. Introduction

Betaine (2-*N*,*N*,*N*-trimethylammonium acetate) is of great physiological importance and can be found in many organisms. In humans it functions as a homocysteine methylating agent, in plants it can act as an osmolyte in case of water or salt stress [1]. Betaine is therapeutically used in the treatment of different forms of homocystinuria, as well as a hepatoprotectant and against arteriosclerosis [2, 3].

Echinacea species (*E. purpurea* and *E. angustifolia*, Asteraceae) are known to contain up to 1.5% of betaine [4], and some manufacturers use this compound to standardize their products. The analysis of quaternary ammonium salts like betaine is difficult, because of their poor UV absorption and retention behavior. Therefore, in most of the previous publications this compound was analyzed by the use of strong cation exchange column materials, with a detection wavelength of 200 nm [4] or 254 nm (as the corresponding bromophenacyl ester) [5, 6]. The detection limit for the bromophenacyl ester is much lower, but the derivatization procedure can be rather time consuming or includes the use of rare reagents. Since it was not possible to prepare the mobile phase as described, the most recent system could not be reproduced [2].

In this paper we present an improved HPLC method (different mobile phase, modified sample derivatization) for the determination of betaine, which combines an easy and fast sample preparation with high sensitivity. The practical use of this method is demonstrated by analyzing different *Echinacea* samples and market products.

2. Investigations and results

2.1. Method Development

A direct and sensitive UV detection of betaine is hardly possible even at short wavelengths, because only a weak chromophore (carbonyl group) is present in the molecule. Therefore, in order to lower the detection limit, betaine was quantified as its 4-bromophenacyl ester. Betaine was derivatized according to literature procedures [2], with some modifications (increased concentration of 18-crown-6, higher temperature and larger volumes of sample- and phosphate solution). These changes allowed a quantitative derivatization of the compound within 30 min at 90 °C, which is a time reduction of 50% compared to the previously published methodology.

Betaine is a very polar compound and therefore hardly retained by reversed phase material. Thus, a satisfactory separation with this stationary phase is not possible, especially if plant extracts are to be analyzed. The ionic properties of betaine can be utilized if a separation by ion exchange chromatography is possible. For this technique choline has been reported to be a useful counterion in the elution of quaternary ammonium compounds [1]. Studying the effect of pH and ionic strength of a choline buffer on the separation of betaine, a pH decrease to 3.5 and an increase of the ionic strength resulted in significantly reduced retention times. Values lower than pH 3.5 did not change the retention behavior of the 4-bromophenacyl ester of betaine. Therefore a 50 mM choline buffer, with a pH of 3.5 was chosen. The buffer was prepared with choline chloride instead of the free base, because of a more convenient handling and an increased stability of the reagent. Another major improvement was the use of 70% methanol in the mobile phase. Compared to acetonitrile the separation and peak symmetry could be enhanced without increasing the total separation time of 12 min.

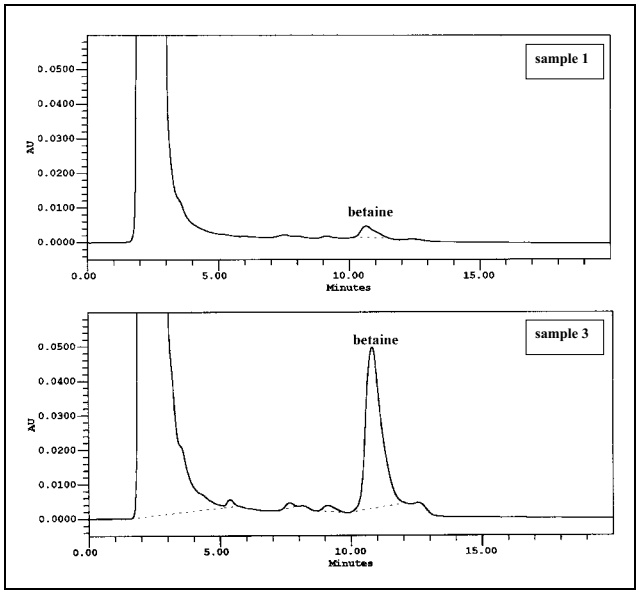

Fig. 1: Chromatogram of two *Echinacea* samples (sample 1 and 3) separated by ion-exchange chromatography (column: Supelcosil LC-SCX, 250 × 4.6 mm, particle size 5 μm; mobile phase: isocratic with methanol/50 mM choline buffer pH 3.5 = 7/3; flow rate 1.5 ml/min; detection: 254 nm; injected sample volume: 10 μl; temperature: ambient).

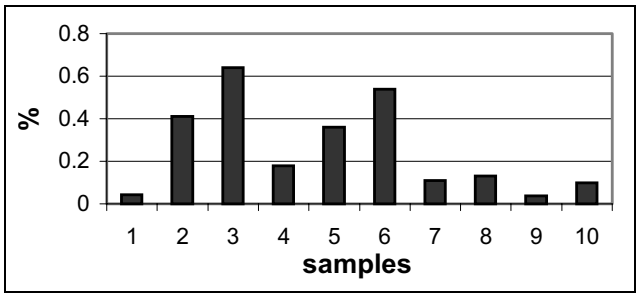

Fig. 2: Variation of the betaine content (in g per 100 g preparation) in different *Echinacea* samples; each was injected 4 times, with standard deviations below 2.5% for all samples.

2.2. Method validation

For calibration experiments betaine standard solutions were prepared with concentrations from 100.0 to 3.1 μg/ml. The correlation coefficient (R^2) was 0.998, with a regression equation of $y = 3.19 \times 10^4 x$. Thus, a linear relationship between absorbance and concentration can be concluded. The lower limit of sensitivity is the limit of detection. It is defined as the concentration where a signal has at least three times the height of the baseline noise. With the developed HPLC method betaine concentrations as low as 0.2 μg/ml fulfilled this requirement. An indicator for precision is the standard deviation (δ). All samples were injected four times and a maximum δ-value of 2.5% was observed. Peak purity was confirmed by studying the PDA spectra of the peak of interest; no indications for peak impurities were observed. Accuracy of the method was confirmed by spiking one *Echinacea* sample (sample 1) with 500 μl of the standard stock solution. The sample was extracted and derivatized as described below (see 4.3 and 4.4), and a recovery rate of 100.4% ($n = 4$, $\delta = 1.2\%$) was obtained.

2.3. Samples

Ten different *Echinacea* market products (solid and liquid formulations) were analyzed (Fig. 1 shows samples 1 and 3). All samples contained betaine at ratios between 0.04 and 0.64% (Fig. 2). Only one sample claimed a certain content of betaine (sample 6) and this value could be verified. In a second study, swimmers of the Spanish Olympic team were treated with *Echinacea* preparations to improve their performance. Blood samples of the athletes were analyzed by the same procedure as the *Echinacea* samples, but betaine could not be determined in any of the samples. The signal was either absent or too insignificant for a clear assignment.

3. Discussion

The method was successfully used for a sensitive and reliable determination of betaine in *Echinacea*. Different specimens, consisting of root, herb or plant extract were analyzed, and the marker compound was assignable in all of them. Product to product variations in the betaine content of more than 15 fold were observed. The attempt to quantify betaine in human plasma samples failed, as the amount of the compound in these samples was too low to be detected. Finally it can be concluded that because of the simple sample preparation, short analysis time and high reproduciblity, the method presented in this paper can be a useful tool for routine analysis of betaine in plant material as well as in commercial preparations.

4. Experimental

4.1. Chemicals

Betaine monohydrate, choline chloride, phosphoric acid and all other reagents (18-crown-6, 4-bromophenacylbromide, potassium dihydrogenphosphate) were bought from Sigma (St. Louis, MO, USA). Solvents (acetonitrile, methanol and water) were of HPLC-grade and purchased from Fisher Scientific (Fair Lawn, NJ, USA).

4.2. Chromatography

HPLC experiments were performed on a Waters HPLC system, equipped with a 996 photodiode array detector, a 712 WISP autosampler and a 600E system controller. An optimum separation was achieved with a Supelcosil LC-SCX column (250 × 4.6 mm; 5 μm particle size) from Supelco (Bellefonte, PA, USA), using a mobile phase consisting of methanol/choline buffer (7/3). The choline buffer was prepared by dissolving 50 mM choline chloride in water, adjusted to pH 3.5 by the addition of 10% aqueous hydrochloric acid. 10 μl of the sample and standard solutions were injected, respectively; the flow rate was adjusted to 1.5 ml/min, with a detection wavelength of 254 nm. The separation was performed at ambient temperature.

4.3. Preparation of standards and samples

Standard stock solution: 10.0 mg of betaine monohydrate was dissolved in 10.0 ml of 50% aqueous methanol. Further calibration levels were prepared by diluting the stock solution with the same water/methanol mixture, and the resulting range of concentration was from 100.0 to 3.1 μg/ml (based on the monohydrate).
Echinacea samples: Ten commercial products (capsules, tablets, and liquids), consisting of *E. angustifolia*, *E. purpurea* and mixtures thereof were analyzed. The samples were bought in a local supermarket in Oxford/MS, and voucher specimens are deposited at the NCNPR.
1.000 g of a solid sample (samples 1–8) was extracted three times with 3 ml of 50% aqueous methanol. After sonication for 10 min and centrifugation at 3500 rpm the supernatants were combined in a 10 ml volumetric flask and filled up to the final volume with extraction solvent. Liquid samples (samples 9 and 10) were used directly without any dilution.
Plasma samples: Plasma was used directly.

4.4. Derivatization procedure

Reaction mixture: 50 mM 4-bromophenacyl bromide and 25 mM 18-crown-6 in acetonitrile. This solution is stable for about 4 weeks, if stored at 4 °C.
Potassium phosphate solution: 100 mM of KH_2PO_4 in water.
Preparation: 100 μl of standard, sample solution or plasma and 100 μl of potassium phosphate solution were placed in a 1 ml vial and mixed. After adding 800 μl reaction mixture, the solution was shaken again, and the closed vial kept at 90 °C for 30 min in a heating block. Prior to HPLC the solution was allowed to cool to room temperature and filtered through a 0.45 μm Acrodisc membrane filter (Gelman, Ann Arbor, MI, USA).
The derivatives were stable for at least 4 weeks, if stored at 4 °C.

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