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Note

Chromatographic analysis of choline and acetylcholine by UV visualization

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In the last decade considerable interest has been generated in the role of choline and acetylcholine in plants. Several methods exist for the detection of these compounds after extraction from biological tissue: bioassay[1], spectrophotometry[2–4], and gas[5–7], liquid[8,9], and paper[10] chromatography. However, all these methods have certain disadvantages. Bioassays and spectrophotometric procedures may be sensitive, but they are not necessarily specific. Gas chromatography of choline esters requires a demethylation procedure before they become volatile for analysis. Demethylation may be accomplished by either a lengthy chemical demethylation procedure using sodium benzenedithiolate[11] or by direct pyrolysis of the sample[6]. Both types of demethylation procedures have been reported to cause cleavage of the ester moiety from choline leading to potentially erroneous results[12,13]. Liquid chromatographic procedures require pre- or post-column derivatization of choline esters for detection and quantitation. Separation of choline and choline esters by paper chromatography is laborious and can be quantitatively inaccurate.

Because of the physiological significance of choline and acetylcholine esters in plant and animal studies[1,14–16], an improved method for separating and quantifying these compounds is needed. Reversed-phase high-performance liquid chromatography (RP-HPLC) provides unique advantages over the above techniques. It is rapid, reproducible, offers high resolution, and is quantitatively accurate. In addition, chemically unaltered compounds may be collected from the system after column separation and stored for further analysis. When coupled with UV-visualization methods, RP-HPLC obviates the need for derivatization of choline and choline esters prior to analysis. Briefly, UV visualization allows one to quantify non-UV-absorbing ionic compounds with a UV-sensitive detector by the addition of UV-absorbing ion-interaction agents to the mobile phase. When a sample is applied to the column, a pair of ions (consisting of a non-UV-absorbing analyte ion and a UV-absorbing counter ion) migrates through the column. The UV-absorbing ions are in a dynamic equilibrium between those adsorbed to the stationary phase and those in the mobile phase. A pair of ions (not an ion pair) will eventually coelute and is detectable in the UV range. The analyte ion and the UV-absorbing counter ion may be the same or opposite in charge. The UV-absorbing ion-interaction agents are locally desorbed from the stationary phase by injection of analyte ions of the same charge; UV-absorbing ion-interaction agents are locally adsorbed by injection of analyte ions of

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opposite charge[17]. Ion-interaction and UV-visualization chromatography mechanisms are the subjects of extensive reviews by Bidlingmeyer[18], Bidlingmeyer *et al.*[17], and Stranahan and Deming[19].

Application of UV visualization in RP-HPLC systems is still relatively new, and it has only recently appeared in the literature. In the current study UV-visualization RP-HPLC is used to analyze choline, acetylcholine, and two related choline esters. The objective was to provide simple, rapid techniques for the analysis of choline, acetylcholine, and related choline esters in a system requiring little or no sample preparation.

EXPERIMENTAL*

Instrumentation

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system equipped with a Model 6000A solvent delivery system and a Model U6K universal injector were used with a Radial-Pak μBondapak C_{18} cartridge in a Waters RCM-100 radial compression module. Detection was by means of a Waters Model 440 absorbance detector with a 254-nm wavelength kit. All analytes used in the study produced positive peaks and the retention times and peak areas were obtained from an Autolab System I computing integrator (Spectra-Physics, Santa Clara, CA, U.S.A.). The Autolab did not sense negative peaks.

Chemicals

Acetylcholine chloride (Ach), butyrylcholine chloride (Bch), choline chloride (Ch), and propionylcholine chloride (Pch) were obtained from Sigma (St. Louis, MO, U.S.A.). High-purity glass-distilled solvents (butanol and methanol) were obtained from MCB (Cincinnati, OH, U.S.A.) and buffer-grade acetic acid came from Pierce (Rockford, IL, U.S.A.). 1-Phenethyl-2-picolinium bromide (PEP) was obtained from Eastman-Kodak (Rochester, NY, U.S.A.). A 10 m*M* PEP stock solution was prepared prior to addition to the mobile phase. The stock solution was extracted once with dichloromethane to remove UV-absorbing impurities and was stored in refrigeration for no longer than two weeks.

Separation procedures

A wide variety of solvents ranging from pure deionized water to 100% methanol, were tested with PEP. Optimum separation occurred with 0.15 m*M* PEP in butanol–methanol–acetic acid–water (8:4:2:86). The effects of variable PEP concentration (0.05 and 0.10 m*M*) were also evaluated in this system.

Generally, the passage of 150–200 ml of mobile phase through the system was required before equilibrium was established. Background absorbance was offset electronically.

Choline and choline ester standards (4.0 m*M*) were prepared in deionized water. Samples of 10 μl were chromatographed alone and in combination. Variable

* Mention of a trademark name or proprietary product does not constitute endorsement by the University of Arkansas Experiment Station and does not imply its approval to the exclusion of other products that may also be suitable.

concentrations of Ach and Ch were applied in equal volume (10 μl) injections in order to evaluate the response of the detector and from these data standard curves were generated. Choline and acetylcholine were detectable to the 1-nmol level in this system.

Calculations

The following chromatographic parameters were calculated from the retention times: k', the relative capacity factor; α, the separation factor (relative retention); R_s, the resolution; and N, the theoretical plate number of the columns. The following equations were used[20]:

$$k' = \frac{t_1 - t_0}{t_0}, \qquad \alpha = \frac{k'_2}{k'_1}, \qquad R_s = \frac{t_2 - t_1}{1/2(w_2 + w_1)}, \qquad N = 16\left(\frac{v}{w}\right)^2$$

where t_1 = retention time of component 2, t_1 = retention time of component 1, t_0 = retention time of non-adsorbed solvent, v = retention volume, w_2 = the peak width in seconds of component 2, w_1 = the peak width in seconds of component 1, k'_2 = capacity factor of component 2, and k'_1 = the capacity factor of component 1. For Waters Radial-Pak μBondapak C_{18}, $N \approx 5000$ plates.

Plant extract

The uppermost fully expanded leaves of greenhouse-grown V5 "Davis" soybeans were excised and immediately frozen at −20°C in a freezer. A 4-g fresh weight of frozen tissue was placed in a 50-ml centrifuge tube containing 25 ml of acetonitrile–0.4 *M* formic acid (85:15) and homogenized on a Willems Polytron (Brinkman, Westbury, NY, U.S.A.) for 1 min in an ice bath. The homogenates were allowed to stand for 1 h in the ice bath and then were centrifuged at 5000 *g* for 10 min at 4°C. After centrifugation, the supernatant was decanted into a 100-ml centrifuge tube and stored on ice. The pellet was resuspended in 25 ml of acetonitrile–0.4 *M* formic acid (85:15), vortexed, allowed to stand on an ice bath at 4°C for 1 h to precipitate relatively water-insoluble components, and centrifuged at 5000 *g* for 10 min. The supernatants were combined and the pellets discarded.

To the combined supernatants was added one volume of a mixture of toluene–ether (1:1). The tube was vortexed, centrifuged at 150 *g*, and the upper organic layer was aspirated and discarded. The solvent extraction was repeated twice. The aqueous extract was again allowed to stand on ice for 1 h to precipitate the remaining relatively water-insoluble components. After 1 h the extract was centrifuged and the supernatant was carefully pipetted to a clean test tube. The volume of the aqueous extract was approximately 3 ml.

The extract was spiked with 400 nmol each of acetylcholine, choline, and propionylcholine and then passed through a Waters C_{18} Sep-Pak cartridge. The eluent was collected and saved. The cartridge was further eluted with nine, 3-ml volumes of mobile phase containing 0.15 m*M* PEP. All ten 3-ml fractions were then frozen, lyophilized, and the residues resolubilized in 200 μl of mobile phase. A 10-μl sample of each fraction was analyzed by HPLC using the mobile phase containing 0.15 m*M* PEP. These samples were compared against 400-nmol standards dissolved in water and eluted through a Sep-Pak in an identical manner. Plant extract and standard elutions were replicated three times.

RESULTS AND DISCUSSION

Analysis of standards

The chromatograms presenting the separation of choline esters with three concentrations of PEP on μBondapak C_{18} are presented in Fig. 1. All analyte ions produced positive peaks followed by one negative system peak. The retention time of the large negative peak following the analyte peaks coincides with the retention time of PEP when it is injected as a sample.

When a positively charged sample analyte is applied to the column PEP is desorbed from the stationary phase and coelutes at the apparent retention time of the analyte. In a four-component sample (Ach, Bch, Ch, Pch) the amount of PEP desorbed by an analyte and subsequent detector response is apparently inversely proportional to the polarity of that analyte (Ch < Ach < Pch < Bch). All four analytes were detectable regardless of PEP mobile phase concentration although detector response decreased with PEP concentration. The most polar analyte (Ch) eluted first, the least polar (Bch) eluted last, and then a strongly negative system peak followed. The negative system peak occurs immediately following the Bch peak when either 0.10 m*M* or 0.15 m*M* PEP is present in the mobile phase. The potential for overlap between the two peaks may result in errors in the quantitation of Bch. The negative system peak represents the amount of PEP desorbed from the column by the analyte ions[19]. The greater the concentration of analytes in the sample, the larger the system peak will become, and there is a greater potential for overlap between the system peak and the analyte peak(s) immediately preceding or following it. This source of error may be overcome by reducing the concentration of analytes in the sample, applying a smaller sample volume to the column or by solubilizing the sample in mobile phase containing PEP. This situation may not necessarily be a problem since positive peaks can occur within the area of the negative system peak (Fig. 2).

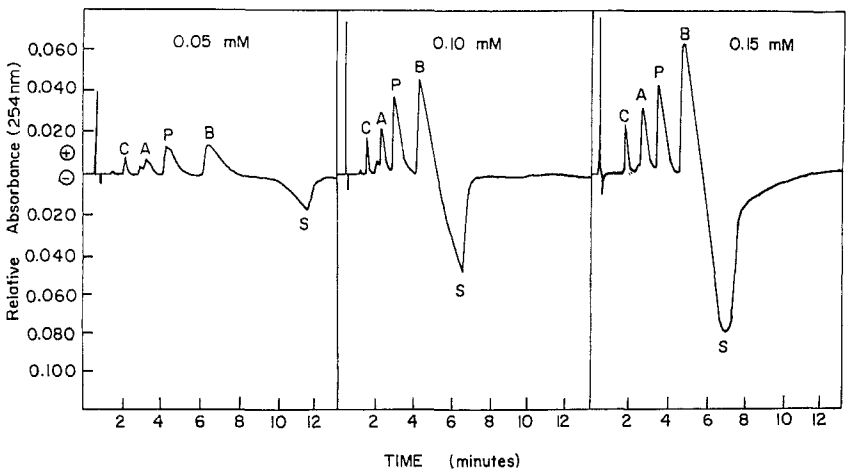

Fig. 1. Chromatograms of 40-nmol equimolar mixtures of acetylcholine (A), butyrylcholine (B), choline (C), and propionylcholine (P) using three different mobile phase PEP concentrations; system peak (S).

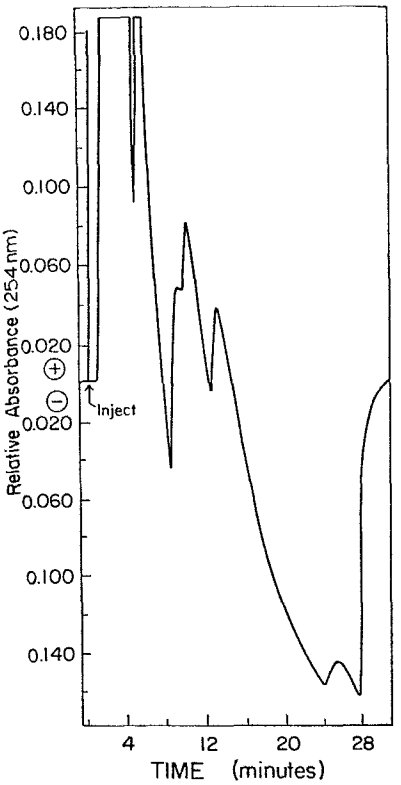

Fig. 2. Chromatogram of a soybean leaf extract; fraction 1 of a 3-ml C_{18} Sep-Pak elution (10-μl sample injection).

A properly programmed integrator may compensate for the apparent changes in the baseline and provide accurate positive peak areas.

Table I presents the retention times and resolution data for each of the mobile phase PEP concentrations. The retention times for all analyte ions were inversely related to PEP concentration in the mobile phase. The k' values for all analytes were within or near the optimal 1–10 range with the highest PEP concentration only. Both Bch and Pch with 0.05 mM PEP in the mobile phase and Bch with 0.10 mM PEP in the mobile phase fell well outside the optimal k' range. All R_s values were >1.0 except for choline with the lowest concentration of PEP in the mobile phase.

The mobile phase of choice for this system would be that containing 0.15 mM PEP. This system offers reduced retention times, enhanced detector response, and adequate k', α, and R_s values for all of the analytes examined.

Detector response to variable concentrations of Ach and Ch is presented in Table II. Response to increasing concentrations of Ach and Ch (5–100-nmol range) was linear in both systems and was proportional to the amount of analyte present. Concentrations as low as 1 nmol could be detected.

Analysis of a leaf extract

Approximately 77% Ch, 73% Ach, and 78% Pch were recovered from the first

TABLE I

RETENTION TIMES AND CHROMATOGRAPHIC PARAMETERS FOR THE SEPARATION OF CHOLINE DERIVATIVES ON RADIAL-PAK A C_{18} USING THREE CONCENTRATIONS OF PEP IN THE MOBILE PHASE

Mobile phase: butanol–methanol–acetic acid–water (8:4:2:86); flow-rate: 3 ml/min.

*Standard**	*Retention** time (sec)*	t_0 = 48 sec		
		k'	α	R_s
0.05 mM PEP				
Choline	470	8.79	1.12	0.87
Acetylcholine	520	9.83	1.37	2.15
Propionylcholine	695	13.47	1.54	2.64
Butyrylcholine	1004	20.75		
0.10 mM PEP				
Choline	248	4.17	1.66	3.45
Acetylcholine	381	6.94	1.34	1.79
Propionylcholine	493	9.27	1.51	2.14
Butyrylcholine	719	13.98		
0.15 mM PEP				
Choline	208	3.33	1.65	2.29
Acetylcholine	312	5.50	1.35	1.25
Propionylcholine	404	7.42	1.42	1.24
Butyrylcholine	554	10.54		

* A concentration of 40 nmoles each in 10-μl injections.
** Mean of three injections.

two fractions of a mixed standard eluted from the C_{18} Sep-Pak with mobile phase, when compared against direct injection of the choline esters. Choline esters, if present, were below the limits of detection in fractions 3–10.

Development of a procedure for the quantitative extraction of Ach and Ch from plant tissue was not successful. Our extraction procedure was based upon those

TABLE II

PEAK AREA RESPONSE TO VARIABLE AMOUNTS OF CHOLINE AND ACETYLCHOLINE

Stationary phase: μBondapak C_{18}; mobile phase: butanol–methanol–acetic acid–water (8:4:2:86); flow-rate: 3.0 ml/min.

Nanomoles	*Choline*		*Acetylcholine*	
	*Peak area units**	*C.V. (%)*	*Peak area units**	*C.V. (%)*
5.0	4351	5.2	8973	1.5
12.5	12032	0.9	23557	2.7
25.0	22889	3.1	46067	1.4
50.0	44715	1.0	90872	0.5
100.0	86442	0.4	178970	0.8
r	0.9997**		0.9999***	

* Mean of three injections.
** Significant at 0.01 level.
*** Significant at 0.001 level.

in the literature that reported successful choline and choline ester extraction[5,7]. Choline and choline esters present in the leaf extract were not detectable. Fig. 2 presents the chromatogram of a 10-μl sample from fraction 1 of a soybean leaf extract eluted through a C_{18} Sep-Pak. Fraction 2 presented a chromatogram similar to fraction 1. The presence of interfering substances that interact with PEP, and that are probably also strong UV-absorbing components, severely inhibits the ability to separate and quantify choline and choline esters from leaf tissue extracts. The interfering substances are probably phenolic compounds that are known to be present in substantial amounts in plant tissues[21].

To date few investigators have reported successful analyses of Ach and Ch from plants. The methods that have been reported are subject to the same limitations described in the introduction: low specificity, potential cleavage of choline esters, and/or unwieldy derivatization procedures which may also lack specificity. The phenolic components of plants may interfere with many of the methods reported.

The technical difficulties in purifying a plant extract sufficiently for quantitative analysis by UV-visualization RP-HPLC have yet to be overcome. In general, the difficulty in quantitatively extracting Ach and Ch from plant tissue may be one reason for the relative paucity in research concerning Ach physiology in plants.

Application of UV visualization with RP-HPLC provides a new method for analyzing Ach, Ch, and related choline esters. No pre- or postcolumn derivatization is required for the detection and quantitation of these analytes after quantitative extraction from biological tissue.

Procedures for the quantitative extraction of Ach and Ch from plant tissue are currently being refined. These investigations will be the subject of a future report.

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