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RAPID REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTITATION OF VICINE, CONVICINE AND RELATED COMPOUNDS

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SUMMARY

A high-performance chromatographic assay for vicine and convicine in fababeans and tissue extracts and for the qualitative estimation of the unstable breakdown products of the aglycone form of these compounds has been developed. The method makes use of a reversed-phase system with a C_{18}-bonded column and yields quantitative recovery of both compounds. This procedure is superior to other analytical techniques currently available because of simplicity of sample preparation and the sensitivity and rapidity of the method.

INTRODUCTION

Vicine [2,6 diamino-4,5-dihydroxypyrimidine, 5-(β-glucopyranoside)] and convicine [2,4,5-trihydroxy-6-aminopyrimidine, 5-(β-D-glucopyranoside)] are present in fababeans (*Vicia faba* L.)[1–3]. The aglycones (divicine and isouramil) of these compounds have been implicated in the human disease, favism[3–6]. Vicine, when consumed by laying hens causes a marked increase in level of lipid and lipid peroxides and degree of erythrocyte hemolysis[7].

Previous methods for the analysis of vicine and convicine have involved a non-specific colorimetric[8] or ultraviolet[9] test; separation of the compounds using paper or thin-layer chromatography followed by quantitation using ultraviolet spectrophotometry[10–12], or more recently by the use of gas–liquid[13] or amino acid[14] chromatography. These methods are either non-specific or require arduous and time-consuming techniques. The gas–liquid chromatographic method for example requires prior derivatization which is not only time consuming but can lead to incomplete derivatization or the formation of optical isomers. Also procedures for the quantitation of vicine and/or convicine in animal tissues and for the detection of the labile aglycone forms of these compounds has not been reported in the literature.

This paper describes a simple and specific method involving the use of reversed-phase liquid chromatography for separating vicine, convicine and their hydrolytic products. The method is capable of detecting these compounds at concentrations as low as 0.1 μg/ml. Analysis time for elution of these compounds is less than 3 min.

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No pre-treatment is required other than deproteinization. Because of the simplicity of the method, its application may be directly utilized for not only routine screening of various cultivars of fababeans but also for metabolic studies with vicine, convicine and/or their aglycones.

EXPERIMENTAL

Apparatus

The chromatograph consisted of a Beckman Model 110A pump (Beckman, Irvine, CA, U.S.A.) coupled to a ISCO Model UA-5 multiwavelength absorbance monitor (ISCO, Lincoln, NE, U.S.A.). Injection was made by syringe via a Beckman Model 210 injector valve containing a 20-μl sample loop. The high-performance liquid chromatographic (HPLC) stainless-steel column (250 × 4.6 mm I.D.) was prepacked by the manufacturer with ultrasphere ODS (mean particle 5 μm, Beckman). A guard pre-column (40 × 4.6 mm I.D.) was packed with Co:Pell ODS (mean particle diameter 30 μm, Whatman, Maidstone, Great Britain). Absorbance was monitored at 280 nm and peak heights and areas were determined.

Chemicals

Uric acid, cytosine, 1-dihydroxyphenol alanine (DOPA), and tyrosine (Tyr) were from Sigma (St. Louis, MO, U.S.A.). Reagent grade sulphuric acid, phosphoric acid, ammonium hydroxide, perchloric acid (PCA), ethylether and methanol were from Fisher Scientific (Winnipeg, Canada). Analytically pure vicine was prepared by the procedures of Lin and Ling[15] and Olaboro *et al.*[14]. For the latter isolation, two additional recrystallizations were carried out. Convicine was prepared by the procedure of Bien *et al.*[2] and the procedure of Marquardt *et al.*[16]. The aglycones of vicine and convicine was prepared by the method of Lin and Ling[15].

Preparation of reagents

Standard solutions of vicine and convicine were made up as 1.08 (328 μg/ml) and 0.49 (150 μg/ml) m*M* stock solutions and were prepared by dissolving appropriate masses of the dried standards in water. The stock solutions of these compounds were stored at 2°C for 1 week. The standard stock solutions of other compounds were 1 m*M* in concentration and were prepared fresh daily in water. Prior to analysis the stock solutions were diluted to the appropriate concentrations with either water or PCA. Stock solutions diluted in 50% PCA were prepared each day and were maintained at temperatures of between 2 and 6°C so as to minimize hydrolysis of vicine and convicine.

Finely ground fababeans, fababean fractions or whole tissue samples were diluted with 100 volumes or less of 5% PCA. The fababean samples were mixed for 10 min at 20°C and tissue samples were homogenized for 30 sec in an ice bath using a Polytron PT-10 homogenizer (Brinkmann, Westbury, NY, U.S.A.). Blood samples were precipitated with 4 volumes of 6% PCA. The extracts were centrifuged at 13,000 *g* for 10 min, and the supernatants were filtered (13 mm cellulose acetate, 0.45 μm pore size, Millipore, Bedford, MA, U.S.A.). The supernatants were diluted as required with 5% PCA and maintained at 2–6°C until analyzed. Liver samples, prior to being filtered were extracted with water-saturated ethylether. All samples were ana-

lyzed within 6 h of preparation. Statistical analyses were carried out according to the procedures of Snedecor[17].

The stock eluting solution (0.5 *M*) which was stable for several weeks at 2°C was prepared from concentrated phosphoric acid and distilled–deionized water and was titrated with ammonium hydroxide to a final pH of 1.44). This solution when diluted 10 fold with water was used as the eluting solution (0.05 *M*). The eluting solution which had a final pH of 2.0 was stable at 23°C for one week. Upon completion of daily analysis, the column was washed with water, a mixture of methanol–water (70:30) and if necessary with methanol. Prior to using, the column was flushed with water and was equilibrated with the eluting buffer. All aqueous containing solutions were passed through a 0.45 μM aqueous filter (Millipore). Methanol was filtered through a 5 μM polytetrafluoroethylene filter. Eluents were degassed by filtration or in an ultrasonic bath (Mettler Electronic Corporation, G.F.R.).

RESULTS AND DISCUSSION

Optimal conditions

The chormatogram in Fig. 1A shows the separation of vicine, convicine and other compounds. These results demonstrate that free cytosine which is not present or may be present in relatively low concentrations in fababeans and in certain tissue extracts can be employed as an internal standard. DOPA and Tyr, two ultraviolet absorbing compounds that are present in fababeans[18], are resolved into two distinct peaks. Uric acid which is present in high concentrations in avian liver and kidney tissues also does not co-elute with either vicine and/or convicine. The injection solvent (5% PCA) is responsible for the small initial peak. The average elution time for all compounds was 8 min and that of vicine and convicine 2 and 2.7 min, respectively.

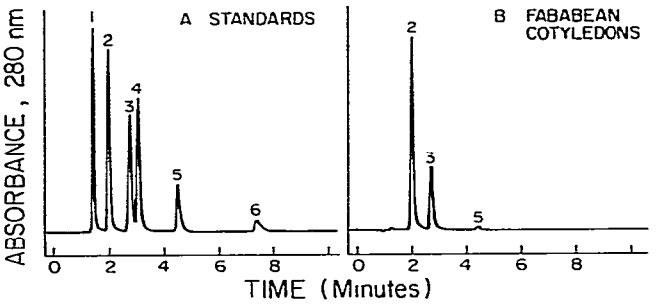

Fig. 1. Chromatogram of a standard solution (A) containing: (1), 0.17 m*M* cytosine; (2), 0 17 m*M* (51 μg/ml) vicine; (3), 0.17 m*M* (50 μg/ml) convicine; (4), 0.17 m*M* uric acid; (5), 0.33 m*M* DOPA and (6), 0 33 m*M* Tyr and (B) an extract prepared from the cotyledon portion of fababeans (0.4 g/40 ml) Compounds eluted in chromatogram B were vicine (2); convicine (3); and DOPA (5). Fababeans and the standard solution, were either extracted or prepared in 5% PCA. Injection volume was 20 μl; flow-rate, 2 ml/min; chart speed, 1 cm/min, optical density setting, 0 2 and temperature, ambient See Experimental for further details.

Preliminary studies demonstrated that optimal resolution of vicine and convicine was achieved at low pH levels. Increasing the pH of the eluent to 3.0 decreased elution times of convicine, DOPA and Tyr by 23 to 120% whereas the elution time of vicine was increased by 50%. DOPA and convicine co-eluted at pH 3.0. Other results

demonstrated that the elution patterns of vicine and convicine were variably influenced by the nature of the injecting solution. Injection of vicine and convicine in water, column eluting solution, 1 *N* hydrochloric acid, 1 *N* sodium chloride and 10% PCA yielded chromatograms with nearly identical elution times and peak areas. Sulphuric acid (2 *N*) had only a negligible effect on the elution profile of these compounds. Modified elution patterns were obtained however, when the standards were injected in solutions that contained a 50:50 mixture of water and methanol or ethanol, 1 *N* sodium hydroxide or 5% TCA. Sodium hydroxide yielded distorted peaks and altered elution times. Concentration of less than 0.5 *N* sodium hydroxide caused only slight distortions. Solutions containing methanol and ethanol greatly reduced retention times of all compounds which caused the peaks to merge. Standards or unknown samples can therefore be prepared in the former but not the latter solutions. The low pH of the eluting buffer does not cause destruction of the column as a single column has been used for the analysis of more than 1000 samples over a period of six months.

Stability of vicine and convicine

Preliminary studies demonstrated that aqueous solutions of vicine and convicine, when stored at 2°C, were stable for eleven days or for a period of at least five days when incubated at 26°C. Vicine also does not appreciably decompose when stored over a five-day period at 2°C in 5% PCA; increasing the temperature to 26°C however, decreases its concentration by approximately 40%. Convicine, when incubated in the presence of 5% PCA over a five-day period, had a 30% decrease in concentration at 2°C and was completely inactivated at 26°C. The average decrease in concentration of vicine in the presence of 5% PCA during a 10-h period would be 0 at 2°C and less than 4% at 26°C while the corresponding values for convicine at 2 and 26°C would be approximately 3 and 30%, respectively. These results would suggest that stock solutions of vicine and convicine can be prepared at weekly intervals in water but that working solutions of these compounds should be maintained at 2°C and should be prepared fresh at least every 10 h. Unknown samples should be treated in a similar manner. On the basis of the above results it may be concluded that 5% PCA can be used as a protein precipitant and that filtrate samples can be injected directly into the column. Results under these conditions are identical to those obtained when samples were injected with the eluting buffer.

Linearity of response and reproducibility

The calibration curves for vicine, convicine, DOPA and cytosine at fourteen concentrations over the range of from 600 to 20, 300 to 10, 300 to 10 and 150 to 5 μg/ml respectively were determined. Plots of peak height and area were linear for all compounds ($P < 0.05$) and the value of the intercepts were not significantly different from zero ($P < 0.05$). The correlation coefficients (r) between peak height or peak area and concentrations of the standard compounds were: vicine, 0.991 and 0.995; convicine 0.997 and 0.983; DOPA 0.995 and 0.992; and cytosine 0.998 and 0.994, respectively. Similar values were also obtained when the standards were prepared in water instead of 5% PCA. The lower limits of detection for vicine and convicine were approximately 0.1 μg/ml. The corresponding amount of sample injected into the column was 2 ng. The high sensitivity is partially attributable to the high molar

absorption coefficients of vicine (16,400 at 274 nm)[1] and convicine (15,800 at 274 nm)[2] in acidic solutions.

The average relative standard deviations in peak heights and areas when 40 (n = 6), 4 (n = 6) and 0.4 (n = 6) mg vicine/100 ml PCA were injected were 1.2 and 2.0%. The corresponding deviations for peak height and area when 20 (n = 6), 2 (n = 6) and 0.2 (n = 6) mg convicine/100 ml were injected were 1.5 and 2.5%. Values similar to those of vicine were obtained with DOPA and cytosine. Also similar values were obtained when the diluting solution was water rather than PCA.

Elution profile of fababean and tissue extracts and quantitative recovery

The elution profile of dehulled fababeans (Fig. 1B) demonstrated the presence of two major peaks, vicine and convicine and two minor peaks, DOPA and Tyr. The concentration of vicine and convicine were 0.53 and 0.28% respectively. Treatment of the extract with β-glucosidase and 1% hot sulphuric acid caused the complete disappearance of the vicine and convicine which suggests that other co-eluting compounds were not present in extracts prepared from fababeans. The percent recovery ± S.D. of vicine and convicine from fababean protein concentrate when added at levels equal to these present in fababean protein concentrate were 98.5 ± 2.0 and 96.2 ± 3.4, respectively.

The chromatographic pattern for blood filtrate with and without added vicine and convicine shows that both compounds are readily separated from other eluting compounds (Fig. 2). The percent recovery ± S.D. of vicine and convicine when added at a level of 5, 10 and 20 μg/ml to blood were 98.4 ± 2.1 and 97.6 ± 3.4%, respectively. Similar recoveries were obtained with liver and kidney. Muscle tissue

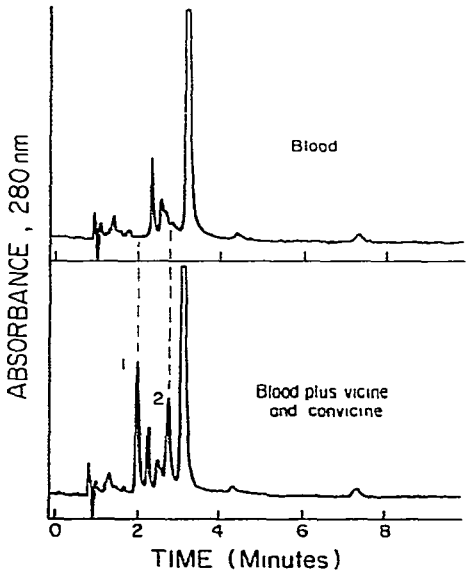

Fig. 2. Chromatograph of avian blood. Upper diagram, control blood; lower diagram, blood plus added vicine (peak 1, 20 μg/ml) or convicine (peak 2, 20 μg/ml) Conditions were as given in Fig 1 or under Experimental, except for the optical density settings which were 0.02.

was not analyzed for recovery as background absorbance levels of the extract were relatively high.

In another study vicine and convicine were fed to chicks at dietary levels of 0.45 and 0.38%, respectively. Analysis for vicine yielded values that were 7, 23 and 37 μg/g, respectively, for blood, liver and kidney. Convicine, in contrast was not detected in any of these tissues. These results would suggest that vicine is absorbed by avian tissue. Convicine in contrast is either not absorbed and/or is metabolized.

Elution profile of hydrolytic products

The results from Fig. 3 show that vicine is rapidly decomposed in hot acid and that during the decomposition at least three different unstable ultraviolet absorbing compounds are formed. After 12 min, vicine was completely hydrolyzed and converted to non-ultraviolet absorbing products. A similar pattern also was observed with convicine. Subsequently studies have demonstrated that the rate of disappearance of these intermediate products is influenced by the concentration of oxygen in solution. The small early eluting peak observed with the upperchromatograms can be attributed to the elution of the injected solvent (2 *N* sulphuric acid). These results would suggest that reversed-phase HPLC can also be utilized as a tool for studying the decomposition of divicine and isouramil *in vitro*.

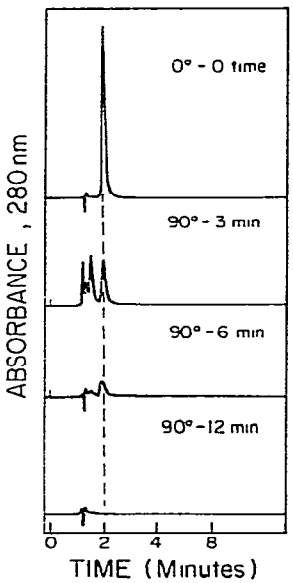

Fig. 3. Chromatogram of vicine and its hydrolytic products Vicine was prepared in 2 *N* sulphuric acid (55 μg/ml) and incubated at 90°C for 0, 3, 6 or 12 min. The samples were immediately cooled in an ice bath and injected Other conditions were as in Fig. 1 or as given under Experimental.

With the reversed-phase separation described, vicine, convicine, their degradation products and other related compounds can be readily quantitated in fababeans and certain tissue samples. This procedure requires a simple sample preparation technique and is sensitive and rapid. We believe that this paper describes for the first time a procedure for the quantitation of vicine in liver, kidney and blood and for the

qualitative detection of degradative products of the aglycone forms of vicine (divicine) and convicine (isouramil). The analytical method described in this paper offers a convenient alternative to the conventional methods currently employed and should greatly facilitate future biological studies involving vicine and convicine and related compounds.

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