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QUANTITATIVE DETERMINATION OF QUINIC ACID AND DERIVATIVES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AFTER DERIVATIZATION WITH p-BROMOPHENACYL BROMIDE

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SUMMARY

Quantitative determinations of quinic, shikimic and glycolic acid have been performed by reversed-phase high-performance liquid chromatography using p-bromophenacyl bromide as a visualizing reagent. The reactions are carried out in N,N-dimethylformamide (DMF) with KF as a catalyst. Linear calibration curves are obtained in the concentration range 0.1–2 mg acid per ml DMF. Spectroscopic and chromatographic properties of the p-bromophenacyl bromide derivatives of quinic, shikimic, dehydroshikimic and glycolic acid are given. A quantitative analysis of quinic acid in plant material is demonstrated.

INTRODUCTION

Quinic, shikimic and dehydroshikimic acid (Fig. 1) are intermediates of the biochemically important "shikimate pathway". They are the precursors of aromatic compounds in plants and micro-organisms[1]. Quinic and shikimic acid are often found in plant materials, in the free acid state or in bound forms with one of their hydroxyl functions esterified to a phenolic carboxylic acid. The amount of free quinic acid can sometimes be as high as 8% on a dry weight basis[2]. Quantitative determination of these acids can be done by high-performance liquid chromatography (HPLC) with refractive index detection[3], low-pressure column chromatography with post-column derivatization[4] or gas chromatography (GC)[5].
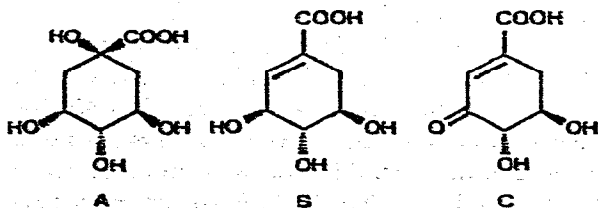

Fig. 1. Acids of the "shikimate pathway": quinic (A), shikimic (B) and dehydroshikimic acid (C).

We looked for a fast and sensitive method which could be used with a reversed-phase HPLC system. p-Bromophenacyl bromide was used successfully for the pre-column derivatization of fatty acids and dicarboxylic acids[6,7]. These reactions either require a tertiary amine as a catalyst, or crown ethers to increase the solubility of the potassium salts. We used KF as a catalyst, as described by Clark and Miller[8], which offers an inexpensive alternative to the above methods. Furthermore, there is no need to convert the carboxylic acids into their salts.

Electron impact (EI) mass spectrometric (MS) analysis of the benzyl derivatives of short chain carboxylic acids provides a convenient means of structure determination according to Politzer et al.[9]. For the derivatives described here, good results were obtained with chemical ionisation desorption (CI/D) MS. EI spectra of these compounds showed only fragmentations from the p-bromophenacyl moiety.

EXPERIMENTAL

Analytical derivatizations

Free acids. N,N-Dimethylformamide (DMF; Merck, Darmstadt, G.F.R.; No. 3039) was saturated with KF at 25°. A 100-μl volume of DMF containing 5 μmole of the organic acid was added to 800 μl of the KF saturated DMF; 10 μmole of p-bromophenacyl bromide in 100 μl DMF, and 100 mg KF, were also added. This mixture was placed in a heating module (Pierce, Rockford, Ill., U.S.A.) at 40° and stirred continuously with a PTFE-coated magnet. After 10 min, 0.5 ml of the reaction mixture were diluted with 2 ml ethyl acetate and 10 μl of the resulting solution were injected into the liquid chromatograph.

Quinic acid lactone. A solution of quinic acid lactone in water (10 mg/ml) was brought to pH 11 for 20 min (pH-stat measurements showed that, at this pH, $k_{hydrolysis}$ is 0.75 min^{-1}). From this stock solution, samples containing from 0.2 to 10 mg of quinic acid were taken. They were injected into a small column (10 × 0.9 cm) of SP-Sephadex (H^{+}) and the quinic acid was eluted with water. This fraction was concentrated to dryness (rotavapor) and dried *in vacuo* over P_2O_5. The dried compound was dissolved in DMF and derivatized as described above.

Syntheses

p-Bromophenacyl dehydroshikimate. 5-Dehydroshikimic acid was synthesized from 1 g shikimic acid by the method of Haslam et al.[10]. After removal of the platinum catalyst by filtration, the reaction mixture was evaporated to dryness and the resulting oil was treated with p-bromophenacyl bromide under the same conditions as described for the analytical derivatizations. p-Bromophenacyl dehydroshikimate was purified by preparative scale reversed-phase chromatography, yielding 220 mg product, m.p. 156°. Molar extinction coefficient 28,100 at $\lambda_{max.}$ = 252 nm.

p-Bromophenacyl shikimate and p-bromophenacyl quinate. A 2.9-mmol amount of acid (quinic or shikimic) and 2.6 mmol p-bromophenacyl bromide were dissolved in 30 ml DMF, 0.5 g KF were added and the reaction mixture was stirred at 40° for 12 min. A 75-ml volume of ethyl acetate was added and the solvents were evaporated to dryness under vacuum. p-Bromophenacyl quinate (shikimate) was obtained from the residue by preparative-scale reversed-phase HPLC. The yield of p-bromophenacyl shikimate was 1.5 mmol, m.p. 138°, molar extinction coefficient 23,900 at $\lambda_{max.}$ =

253 nm. *p*-Bromophenacyl quinate (yield 2.1 mmol) melts at 154°, and has a molar extinction coefficient of 25,600 at $\lambda_{max.} = 254$ nm.

p-Bromophenacyl glycolate. A 1-g amount of glycolic acid and 1 g *p*-bromophenacyl bromide were dissolved in 50 ml DMF; 1 g KF was added, and the reaction mixture stirred at 40° for 20 min. The mixture was then diluted with 100 ml ethyl acetate and filtered. The solvents were evaporated to dryness and water and chloroform added to the residue. *p*-Bromophenacyl glycolate (yield 0.50 g) was obtained from the chloroform phase and washed with cold methanol. It has a m.p. of 156°, and a molar extinction coefficient of 28,100 at $\lambda_{max.} = 252$ nm.

Quinic acid lactone (quinide). Quinic acid lactone was prepared as described by Panizza *et al.*[11].

Mass spectra

General. All mass spectra were recorded on a Riber R10-10B quadrupole mass spectrometer equipped with a System Industries 150 interface, a PDP 8/a computer and a CI/D, EI source. In the chemical ionization mode, the primary ionization of the ammonia reagent gas was accomplished using 70 eV electrons, the pressure in the ion source housing being maintained constant at $2.6 \cdot 10^{-7}$ bar; the pressure in the source itself is then assumed to be *ca.* 1 bar. The source temperature was room temperature for all CI/D experiments and 140° for all GC–MS analyses.

Sample preparation and introduction. (a) CI/D. Using a 10-μl syringe, a drop of a solution containing the sample (*ca.* 100 μg) dissolved in methanol was placed on a non-activated tungsten emitter. The solvent was evaporated from the sample and the probe with the emitter on top was inserted into the reactant gas NH_3. A current of 70 mA was applied to the emitter, and gradually increased to 350 mA at a rate of 8 mA/sec. When ions were observed either on the screen of the computer terminal or on the scope of the mass spectrometer, the current was increased at 1–2 mA/sec.

(b) GC–MS. Plant extracts were trimethylsilylated using Tri-sil (Pierce) in pyridine at room temperature. The silylated extract was separated on a glass column (2.5 m × 2 mm I.D.) filled with 3% SE-30 coated on Chromosorb W. The temperature was programmed from 140° to 230° at 6° min. The helium flow-rate was set at 20 ml/min.

Chromatographic techniques

Analytical separations were performed with a Hewlett-Packard 1080 B liquid chromatograph equipped with a reversed-phase C_8 column (25 cm × 4.6 mm I.D.) filled with LiChrosorb RP-8 (Merck). For preparative scale separations, we used an air-driven pneumatic amplifier pump (Haskel). The preparative column (25 × 2.24 cm) was filled with LiChrosorb 10 RP-8 (Merck). For the purification of the synthesized compounds, appropriate water–methanol eluents were used.

Plant analyses

A 6-g (fresh weight) amount of leaves of *Rosa x Rehderiana* (hybrid *Polyantha*, grafted on *Rosa canina* stock) was extracted with 100 ml 80% ethanol under reflux. This extract was concentrated in vacuum to 5 ml, then centrifuged at 25,000 g for 10 min. The supernatant was filtered over a Centriflo membrane cone (Amicon, Type CF50A). The slightly coloured aqueous phase was injected into a small column

(10 × 0.9 cm) containing polyvinylpyrrolidone (PVP, polyclar AT pract.; Serva, Heidelberg, G.F.R.) as stationary phase.

Non-aromatic polyhydroxy acids were eluted with water. This fraction was poured onto a preparative column (10 × 1.5 cm diameter) of SP-Sephadex C-25 cation exchanger (H⁺) (Pharmacia, Uppsala, Sweden). Organic acids were eluted with water, and the fraction was freeze dried followed by drying over P_2O_5 under vacuum. The residue (0.31 mequiv. of acids) was divided in two parts, which were dissolved in the appropriate solvent and derivatized for analysis by HPLC or GC–MS.

Preparation of the PVP material. A 100-g amount of PVP was boiled in 500 ml 10% HCl for 10 min. The resulting material was filtered off, rinsed with water, washed with acetone and dried.

Determination of the efficiency of the PVP and SP-Sephadex column chromatography. From 1 to 20 mg quinic acid, dissolved in water, were injected in the described columns. The acid was eluted with water and determined colorimetrically after derivatisation as described by Voight and Rawcher[12].

RESULTS AND DISCUSSION

The efficiency of the reaction of *p*-bromophenacyl bromide and the hydroxy carboxylic acids, quinic, shikimic and glycolic acid, was 95, 75 and 87% respectively. The reaction was reproducible, and linear calibration curves were obtained in the concentration range 0.1–2 mg acid per ml DMF. By-products could be formed in small quantities (Figs. 2 and 3). Trace amounts of water reacting with *p*-bromo-
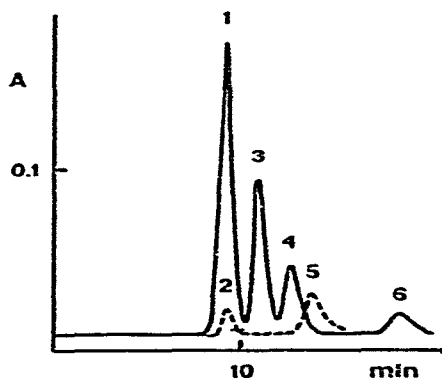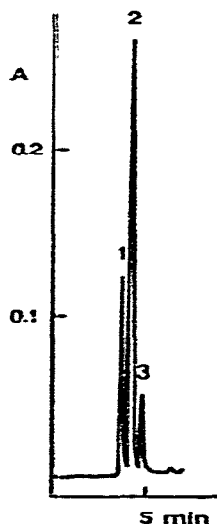

Fig. 2. Reversed-phase gradient chromatography of *p*-bromophenacyl quinate (1), *p*-bromophenacyl shikimate, *p*-bromophenacyl glycolate and *p*-bromophenacyl alcohol (2, unseparated) and *p*-bromophenacyl dehydroshikimate (3). Solvents: A = 5 mM H_3PO_4: B = methanol. Gradient from 35% B to 80% B in 10 min.

Fig. 3. Reversed-phase separation of the *p*-bromophenacyl derivatives of quinic acid (1), glycolic acid (3), shikimic acid (4) and dehydroshikimic acid (6). Compound 2 is *p*-bromophenacyl alcohol; compound 5 is an unknown reaction by-product (separate injection). The eluent is 5 mM H_3PO_4 containing 30% methanol.

phenacyl bromide or with the esters could form some p-bromophenacyl alcohol. Therefore, a blank of p-bromophenacyl bromide and KF in DMF, brought to the same temperature as the reaction mixture during the same amount of time, may be necessary to check whether impurities are present or not. If small amounts of these products are present, the chromatographic conditions could be chosen to avoid their interference with the determination of the investigated compounds.

The four investigated hydroxy carboxylic acids were completely separated by reversed-phase HPLC in an isochratic run, as shown in Fig. 3. p-Bromophenacyl alcohol and p-bromophenacyl quinate were not separated. In a gradient run such as that shown in Fig. 2, the alcohol and the p-bromophenacyl quinate are separated (the variation of the capacity factors, k', with methanol concentration is different for both compounds). The structure of the four p-bromophenacyl esters was verified by ^{1}H-nuclear magnetic resonance, infrared and mass spectroscopy. The classical EI mass spectra showed practically no structural information since no molecular ion $M^{+\cdot}$ was present and all intense fragmentations were derived from the p-bromophenacyl moiety ($m/z = 183, 185, 155, 157$). Therefore, the CI/D mass spectra of these compounds were recorded. This technique readily revealed the molecular weight since the spectra were only characterized by MNH_4^+ ions.

Quantitative determination of polyhydroxy carboxylic acids can be hampered by lactone ring formation. Of the four investigated acids, only quinic acid forms a lactone, the so-called quinide. It was easily converted to the sodium quinate at pH 11 as described under Experimental, and determined with overall efficiencies of 90%. We used a SP-Sephadex cation exchanger (H^+) to convert the sodium quinate to quinic acid before derivatization. Polystyrene resins were tested for the same purpose, but poor recoveries of the acid were obtained. On an Amberlite (AG Type CGII, 40–80 μm; Serva) cation exchanger (H^+), losses from 20 to 40% were observed in a series of quinic acid standards from 20 to 1 mg, injected into a column (10 × 0.9 cm) filled with this material (elution with water). The latter type of ion exchangers are commonly used for the purification of plant extracts. We suggest the use of carbohydrate-based ion exchangers for this purpose (see Experimental). The PVP adsorbents used in the clean-up procedure of plant extracts did not show any irreversible adsorption phenomena, and had good flow characteristics.

We analysed a plant extract with LC and GC. Pre-column derivatisation was used in both analysis, with p-bromophenacyl bromide as described for LC, and with a silylating reagent for GC. Figs. 4 and 5 show the respective chromatograms. Sugars were not removed from the extract, as they normally should not interfere with the LC determination. The GC analysis showed that quinide was not present in the plant extract. Therefore, no additional step (lactone ring opening) was needed in the preparation of the extract for a quantitative determination with HPLC. In the GC determination (Fig. 4), quinic and shikimic acid were recognized on the basis of their retention times and by analysis of the mass spectra of the trimethylsilyl derivatives (with and without CI/D techniques).

Since the molecular ion $M^{+\cdot}$ is completely absent in the EI spectra of these trimethylsilyl derivatives, and major fragment ions $m/z = 73, m/z = 147$ are formed owing to the presence of the trimethylsilyl functions, a GC–MS analysis was performed under chemical ionization conditions with the reactant gas NH_3, to obtain more significant information. Under these conditions trimethylsilylated shikimic and
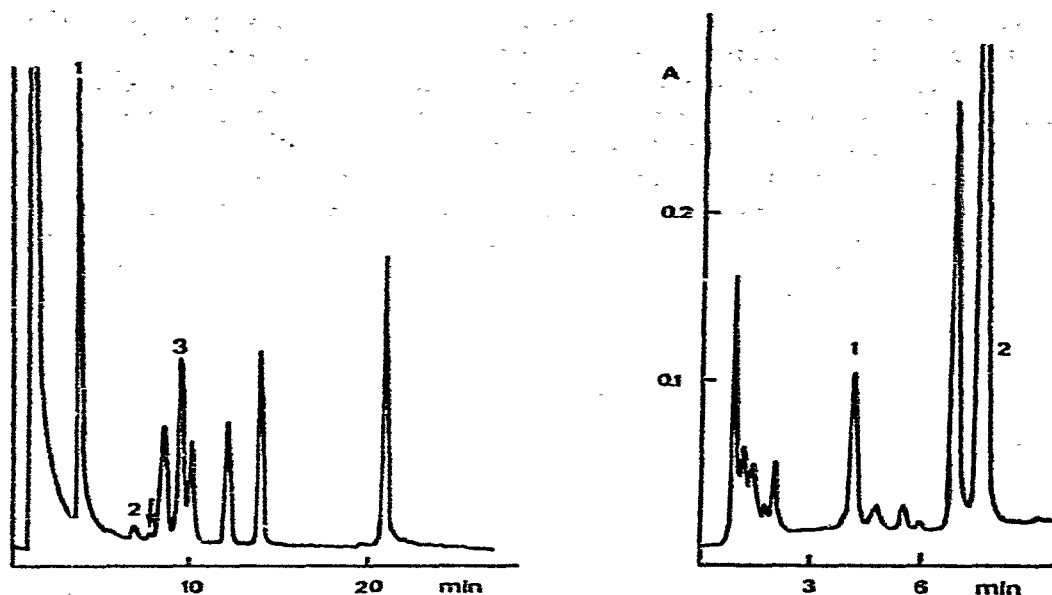
Fig. 4. Gas chromatography of a derivatized extract. The trimethylsilyl derivatives of shikimic acid (2) and quinic acid (3) are present. The arrow indicates the position of the silanized quinide (not present in the extract). Hexadecane (1) was added as an internal standard.

Fig. 5. HPLC analysis of a derivatized plant extract. Peaks: 1 = p-bromophenacyl derivative of quinic acid; 2 = p-bromophenacyl bromide. Column characteristics: see *Chromatographic techniques*. Gradient from 35% B to 80% B in 10 min (A = water, B = methanol).

quinic acid show a MH$^+$ signal at respectively $m/z = 463$ and $m/z = 553$. The ions are the base peak in the spectrum. A quantitative LC analysis of quinic acid (see Fig. 5) gave a value of 0.61 mg per fresh plant leaves.

CONCLUSION

The polar hydroxy carboxylic acids quinic, shikimic and glycolic acid were quantitatively determined using derivatization with p-bromophenacyl bromide in DMF prior to injection into a reversed-phase HPLC system. KF was used as a catalyst. The method was also applied to the analysis of quinic acid in a plant extract. It is shown that the p-bromophenacyl esters of these polar acids are most easily identified by MS with CI/D techniques.

ACKNOWLEDGEMENTS

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