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Short Communication

Simultaneous determination of acetylsalicylic, salicylic, ascorbic and dehydroascorbic acid by HPLC*

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Introduction

It is well known that the stability of acetylsalicylic (ASA) and L-ascorbic acid (AA) in solid dosage forms is generally satisfactory provided that the moisture content is minimized. However, these substances can easily decompose in contact with moisture or in aqueous solution mainly to salicylic acid (SA) and dehydro-L-ascorbic acid (DAA), respectively. An analytical method intended for stability monitoring should therefore be capable of measuring these four chemical entities. Several reversed-phase as well as normal-phase HPLC methods for the separation of ASA and SA have been reported [1–5]. Ascorbic acid has been determined using octadecyl- [6–10] and amino-bonded phases [11]. The use of an octadecyl-bonded phase has been described also for the separation of ASA, SA and AA [12]. Two approaches have been used for the determination of DAA. The first comprises the indirect determination of DAA; both AA and DAA are measured together as total vitamin C after their complete conversion into AA [9, 11] or DAA [13]. In these cases DL-homocysteine was used as the reducing agent and *N*-bromosuccinimide as the oxidizing reagent, respectively. The second approach is the direct determination of DAA after its conversion to a fluorescent compound with *o*-phenylenediamine [10].

In this paper a HPLC method is presented that enables these compounds to be determined rapidly. The method is based on the separation of ASA, SA and AA on an amino-bonded stationary phase using the reversed-phase mode, their detection with a UV detector and the simultaneous fluorimetric determination of DAA after its derivatization into the corresponding fluorophore.

Materials and Methods

Apparatus

The high-performance liquid chromatograph comprised a solvent delivery pneumatic pump (Knauer, 364), a sample injector (Rheodyne, 7125) fitted with a 20-μl loop, a variable wavelength UV monitor (Knauer), a fluorescence HPLC monitor (Shimadzu, RF-535) and a dual channel strip chart recorder (Knauer).

Chemicals and solvents

Acetylsalicylic, salicylic and ascorbic acid were of pharmaceutical purity. KH_2PO_4 and 85% orthophosphoric acid were of analytical-reagent grade. Water was purified by deionization and subsequent distillation. Acetonitrile and methanol (Kemika) were of analytical reagent grade. *o*-Phenylenediamine and *N*-bromosuccinimide were obtained from Fluka.

Chromatographic conditions

The column was a 250 × 4 mm i.d. stainless steel column packed with 10-μm Nucleosil NH_2 (Machery–Nagel). The mobile phase was

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acetonitrile–methanol–KH_2PO_4 (pH 3.5; 0.02 M) (30:20:50, v/v/v). Other buffers of pH 2.5 and 4.5 (0.02 M) were also used. Before use the mobile phase was degassed. The flow rate was 1.2 ml min^{-1}. Column eluents were monitored by a UV detector operating mostly at 280 nm (AUFS 0.04) and by a fluorescence detector with the excitation wavelength set at 350 nm and the emission wavelength set at 430 nm. The range was set to 128 and sensitivity to low. The two detectors were connected in series thus enabling the simultaneous determinations of all four compounds. All experiments were carried out at ambient temperature.

Preparation of solutions

A stock solution of a standard mixture was prepared by dissolving 10.0 mg of ASA, 10.0 mg of AA and 5.0 mg of SA in 25 ml of methanol and diluting the solution to 100 ml with water. After appropriate dilution with water, working standard solutions were prepared containing 0.025, 0.010, 0.005 and 0.002 mg ml^{-1} of SA and 0.05, 0.02, 0.01 and 0.004 mg ml^{-1} of ASA and AA. Working standard solutions of DAA were prepared from the corresponding working standard solutions of AA; 1 ml of 0.05% *N*-bromosuccinimide solution and 1 ml of 0.05% *o*-phenylenediamine solution were added to 5 ml of AA working standard solution and the solution was diluted to 10 ml with water. By this method, working standard solutions containing 0.025, 0.010, 0.005 and 0.002 mg ml^{-1} of DAA were obtained.

A 0.05% *N*-bromosuccinimide solution was prepared by dissolving 25 mg of *N*-bromosuccinimide in 50 ml of water. The solution was stored in a brown bottle in a refrigerator.

0.05% *o*-phenylenediamine solution (OPDA) was prepared by dissolving 25 mg of *o*-phenylenediamine in 50 ml of water. The solution was stored in a brown bottle in a refrigerator.

For sample solutions 10.0 mg of ground powder from capsules was weighed into a 100-ml flask, dissolved in 25 ml of methanol, diluted to 100 ml with water and filtered. A 1 ml volume of OPDA was then mixed with 5 ml of filtrate and the solution was diluted to

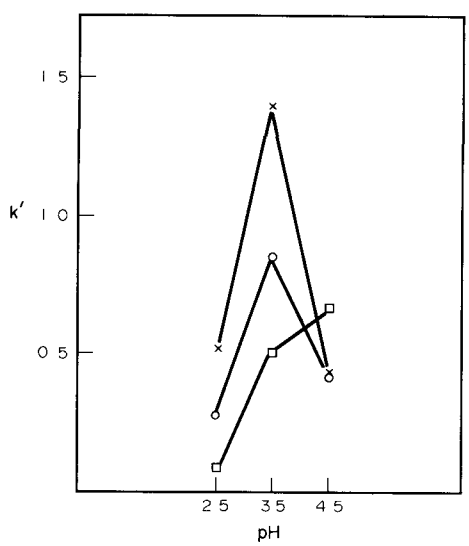

Figure 1
Effect of pH of buffer solutions on the capacity factors k' of the studied compounds. (□), acetylsalicylic acid; (○), ascorbic acid; and (×), salicylic acid.

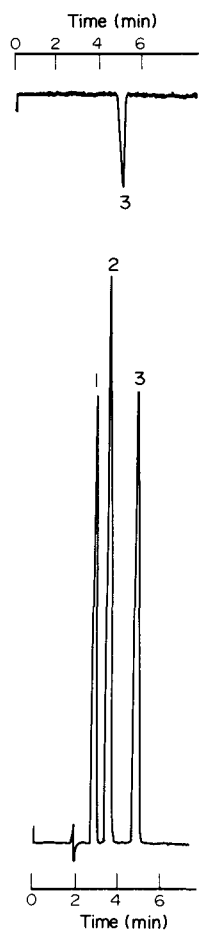

Figure 2
Separation of (1) acetylsalicylic acid, (2) ascorbic acid and (3) salicylic acid under the experimental conditions described in the text; the lower chromatogram denoting UV detection and the upper chromatogram denoting fluorescence detection.

10 ml with water. Solutions were prepared immediately before analyses.

Results and Discussion

The aim of the work was to devise a rapid chromatographic method for the determination of ASA, SA, AA and DAA. The separation of the first three compounds can be achieved using an octadecyl bonded phase but the separation time is long; for this reason, an amino bonded phase operating in the reversed-phase mode was used in the experiments. Although the chromatographic behaviour of the compounds of interest is less predictable with this approach, under the conditions established in this work, rapid and efficient separation has been achieved. As shown in Fig. 1, the pH of the buffer solution used for the preparation of the mobile phase has a great influence on the outcome of separation; coalescence of peaks occurs at higher pH values. The mobile phase composition has similar effects indicating that the presence of acetonitrile is essential for successful resolution of the acid mixture. The resulting chromatogram (Fig. 2) shows that under these experimental conditions SA can also be determined by fluorescence measurements. DAA was measured directly after its derivatization with OPDA to a quinoxaline fluorophore. That the transformation of AA in DAA was complete was confirmed by the absence of the AA peak in chromatograms of AA solutions after the oxidation of AA in DAA [Fig. 3(B)]. OPDA was eluted as a practically unretained compound thus causing no interference with other

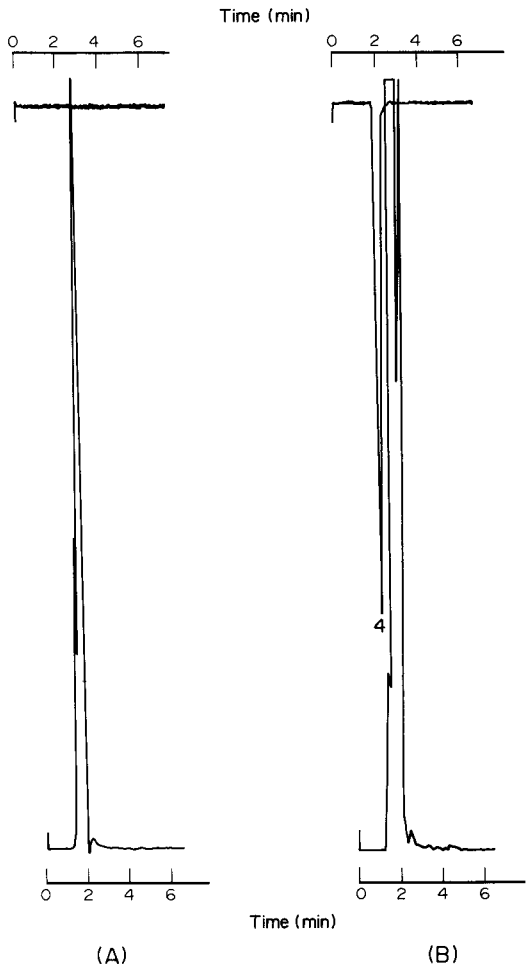

Figure 3
(A) Chromatogram of blank solution after addition of *o*-phenylenediamine solution showing no fluorescence peaks. (B) Chromatogram of (4) dehydro-L-ascorbic acid after its derivatization with OPDA to the quinoxaline fluorophore indicating also the complete transformation of AA in DAA.

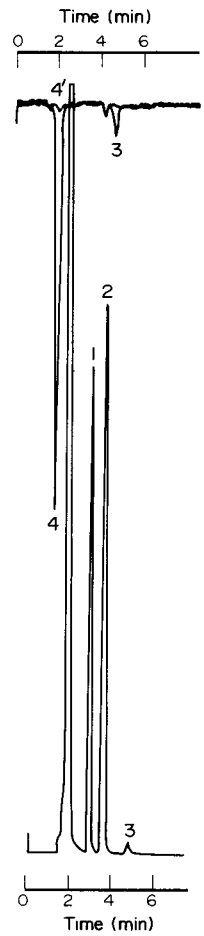

Figure 4
Sample chromatogram of powder from capsules stored for 1 year. Numbers denote the same compounds as Figs 2 and 3. The chromatogram also shows the influence of the sensitivity setting of the fluorescence monitor, (4) at high and (4′) at low sensitivity range.

compounds [Fig. 3(A)]. In addition the fluorescence baseline was not affected and the fluorescence of the slightly retained fluorophore could then be exactly determined [Fig. 3(B)]. Linear responses of the chromatographic method were obtained for all four compounds in the previously mentioned concentration ranges. The correlation coefficients of the corresponding regression lines were greater than 0.99 (12 samples) and the precision of repeated sample injections was about 1% or better (12 samples). The lower limits of detection determined by the confidence interval calculations were about 0.002 mg ml^{-1} or less. These detection limits were obtained using the described experimental conditions, namely UV detection for ASA, SA and AA, and fluorescence detection for DAA. However, the sensitivity can be further enhanced by adjusting the wavelength of UV detection and the sensitivity setting of the fluorescence monitor. This is illustrated in Fig. 4 showing the content of ASA and AA as well as their degradation products in capsules stored for 1 year in the dark at room temperature; fluorescence peaks indicated that about 1% degradation had taken place. In respect of its selectivity, reproducibility and speed, the method proved to be suitable for routine analyses and for stability tests on the compounds studied.

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