Liquid Chromatographic Separation and Simultaneous Analyses of Peroxycitric Acid and Citric Acid Coexisting with Hydrogen Peroxide in the Equilibrium Mixture

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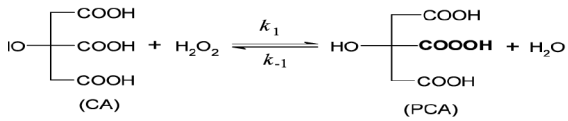
Abstract

The separation of peroxycitric acid (PCA) coexisting with citric acid (CA) and hydrogen peroxide in the equilibrium mixture and their rapid, direct (without derivatization), and simultaneous analyses were successfully carried out with a reversed-phase high-performance liquid chromatographic technique using 3 mM perchloric acid containing 33 mM sodium perchlorate additive as the mobile phase (pH 2.5) and a UV–vis detector. The conditions of separation of PCA from CA were optimized by measuring the chromatograms using different mobile phases containing various additives, with different pHs and temperature. We succeeded in utilizing the narrow (less than one unit) pKa difference between PCA and CA in the present separation process. The selectivity of column in the separation of PCA and CA was also examined. The maximum inter-day coefficient of variation was 8.7% and 9.1% for CA and PCA, respectively. The calibration curves of CA and PCA were constructed and the limits of detection were determined to be 0.41 and 0.13 µM and the corresponding limits of quantification were 1.12 and 0.43 µM, respectively. The mechanism of separation of PCA from the coexisting CA and H_2O_2 was also discussed.

Introduction

Peroxyacids (PAs) have been increasingly used as a disinfectant in hospitals and agriculture, oxidants in organic synthesis and industrial chemical processing, bleaching agent in laundry detergents and cleaning-in-place system in food and beverage industries (1–6). The commonly used PA is peroxyacetic acid (PAA) that coexists with acetic acid (AA) and hydrogen peroxide (H_2O_2) in the equilibrium mixture (1,2). Recently, peroxycitric acid (PCA) has become promising as a hygienic disinfectant (7–9) used in various applications including food preservation (7) and has been synthesized from citric acid (CA) and H_2O_2 [Eq. 1 (6,10)].



Eq. 1

Unfortunately, in analogy with the case of PAA (1–5), PCA could not be isolated from the aqueous reaction mixture containing H_2O_2 and CA (Eq. 1). In addition, CA has three carboxylic (–COOH) groups that may be available for the oxidation reaction by H_2O_2 to form different PCAs (i.e., isomers of PCA). Thus, without the proper assay of each reactant and product in the reaction mixture, the establishment of stoichiometry of the PCA formation reaction and the determination of percent yields are not straightforward.

Many methods, namely UV–vis spectroscopic (12,13), titration (14–16), chromatographic (17–22), conductometric (23), infrared spectroscopic (24–26), potentiometric (27,28), and voltammetric (29–32) measurements have been used to detect and analyze PAs and H_2O_2 in the equilibrium reaction mixture. Based on iodide (I^-) / tri-iodide (I_3^-) redox couple potential buffer, our group has recently developed a sensitive potentiometric technique to determine PAA and H_2O_2 simultaneously in the equilibrium mixture where both PAA and H_2O_2 oxidize I^- to form I^-_3 (27,28). Some research groups have succeeded in employing post- and pre-column high-performance liquid chromatographic (HPLC) derivatization methods for the quantitative analysis of PAA coexisting with H_2O_2 (20–22). All the previously mentioned methods were able to determine total quantity of –COOOH groups present in the corresponding reaction mixture but unable to tell the number of –COOOH groups present in one molecule of PA. On the other hand, the separation of PCA, CA, and H_2O_2 from the reaction mixture would offer their fast analysis as well as authenticate the cleanliness (i.e., information regarding the biproduct formation) of the reaction mixture. The knowledge concerning the products of the PCA formation reaction (Eq. 1) is very important in the practical applications of PCA especially, as a food preservative.

The successful separation of PCA from CA and H_2O_2 coexisting in the equilibrium mixture and their simultaneous analyses

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using reversed-phase (RP) HPLC technique is reported. The choice of the eluting solvent (mobile phase) of the RP-HPLC measurements was achieved using aqueous $HClO_4$ solutions with different additives including sodium perchlorate ($NaClO_4$). The effects of the mobile phase pH, concentration of $NaClO_4$, additive and temperature of the column on the present separation process were investigated. The selectivity of the column in the detection of PCA and CA was also studied, and the calibration curves of CA and PCA were constructed. The mechanism of separation of PCA from CA was extensively discussed.

Experimental

Chemicals and synthesis of PCA

Cautionary note: The mixture of PCA and H_2O_2 is a strong oxidizing agent and in high concentrations may form an explosive mixture (10,33).

All of the chemicals were of analytical grade and the solutions were prepared or diluted with deionized water, Milli-Q, Millipore (Tokyo, Japan). Monohydrated CA (1,2,3-tricarboxylic-2-hydroxy propane, purity 99.5%) and HPLC grade 60% perchloric acid ($HClO_4$), and sodium perchlorate ($NaClO_4$) was purchased from Kanto Chemical Co. Inc., (Tokyo, Japan). H_2O_2 (30%–35%) were available from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). PCA was synthesized according to the procedure described in our previous paper (6).

RP-HPLC apparatus and measurement procedures

The instrumental setup of RP-HPLC measurement has been reported in our previous paper (10). Shortly, the RP-HPLC unit consists of a pump (Model 7410), a UV detector (Model 7450), a column-oven (Model 7432), GL Science (Torrance, CA), an autosampler, Model L-7200, Hitachi, (Tokyo, Japan) and a personal computer for data acquisition with EZChrome Elite, 1997, Scientific Software, Inc., (San Ramon, CA,). The column used was Intersil C8-3 (Torrance, CA) (5 µm 250 × 4.6 mm i.d. and bonded phase is –C8 group). All of the measurements were carried out at a flow rate of the mobile phase of 0.5 mL/min, the column temperature of 25°C and UV detection at 210 nm unless otherwise noted. The column temperature was maintained using an automatic column oven. The injected sample volume was 10 µL. Each measurement was repeated five times for confirming the repeatability. Before each set of RP-HPLC measurement, the column was equilibrated by allowing the mobile phase to run 1.0 h. In this study, the pH of the mobile phase was varied within the range of the recommended limit (pH 2.2–5.5) of the column used (39). A TOA Electronics (Kobe, Japan) model ion meter 1M-55G pH meter was used to measure the solution pH. Before pH measurement, the pH meter was calibrated with the standard buffer solutions with pHs of 1.68, 4.1, and 6.8. The pH of the mobile phase was adjusted to the desired values using dilute NaOH solution.

The retention factor (k') was calculated using the equation of $k' = (t_R - t_0) / t_0$, where t_0 and t_R are the retention times of the unretained (hold-up time) and retained analytes, respectively (38,41). In this work, the retention time of H_2O_2 was assumed as t_0 because no effects of the mobile phase composition, pH and column temperature on the retention time of H_2O_2 were observed.

Determination of calibration curves

A stock solution of CA was prepared by dissolving an appropriate amount of CA crystal in deionized water. The CA stock was standardized by the conventional acid–base titration. The various concentrations of CA solutions were prepared by diluting this stock solution. The calibration curve of CA was constructed by measuring the chromatograms of known concentrations of CA solutions. On the other hand, the reaction mixture containing PCA, CA, and H_2O_2 (Eq. 1), where the concentration of PCA was standardized with conventional cerimetric-iodometric titration (15) and potentiometric (6,27,28) measurement, was used as the source of PCA standard to construct the calibration curve of PCA. The areas of chromatographic peak were integrated by the EZChrome elite software. In each case, the peak areas of the chromatograms obtained with different concentrations were plotted against the corresponding concentrations. The limits of quantitation (LOQ) and the limits of detection (LOD) were determined as ratios of the peak signal by the noise level (S/N = 10) and three times the base line noise, respectively.

Accuracy and precision

The intra-assay precision (coefficient of variation [CV]) and accuracy were evaluated by analyzing the chromatograms measured with five different concentrations of CA and PCA solution on the same day. Similarly, the inter-day assay was examined by analyzing the chromatograms measured on three different days for CA and PCA. In each case, the chromatographic measurements were repeated at least five times.

Results and Discussion

Separation of PCA

Selection of the mobile phase

In the chromatograms shown in Figure 1 measured for the equilibrium reaction mixture (Eq. 1), one well-defined peak at t_R

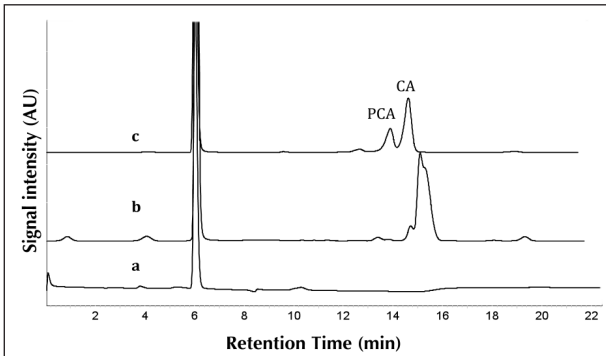

Figure 1. Typical chromatograms measured for the reaction mixture containing PCA, CA and H_2O_2 using mobile phase of acetonitrile–aqueous 3 mM $HClO_4$ (10:90 (v/v) (a), methanol–aqueous 3 mM $HClO_4$ (10:90, v/v) (b), and 3 mM $HClO_4$ in the presence of 33 mM $NaClO_4$ (c). Column temperature: 25°C.

of 6.0 min and a shoulder at t_R more than 16 min (Figure 1a), two well-defined peaks at t_R values of 6.0 and 15.0 min (Figure 1b), and three peaks at t_R values of 6.0, 13.8, and 14.9 min (Figure 1c) were observed. By comparing chromatogram C with those of H_2O_2 and CA measured separately, it was confirmed that the peaks at t_R of 6.0 and 14.9 min are due to H_2O_2 and CA, respectively (10). Thus, the observed peak at t_R of 13.8 min would be ascribed to the product (i.e., PCA) which was confirmed by analyzing this fraction with iodometric titration and potentiometric, electrochemical and liquid chromatographic-mass measurements (10). In fact, the chromatogram shown in Figure 1c was measured at the optimized conditions (described later) where $NaClO_4$ was used as an additive. It is also mentioned that no well-separated peaks for PCA and CA were obtained in the presence of methanol (Figure 1b) and acetonitrile (Figure 1a) additives in the eluting solvent.

When the chromatographic measurement of the reaction mixture (i.e., Figure 1c) was allowed to run for a long time (typically 1 h), no additional peak was found, which indicated that the reaction of CA and H_2O_2 (Eq. 1) produced only one kind of peroxy product, PCA (10). Thus, the negligibly small peaks observed, other than the peaks of CA, PCA, and H_2O_2 are not due to the peroxy compounds (isomers of PCA) as justified with potentiometric measurements, but may be associated with some unknown compounds formed by the decomposition of PCA or CA. Thus, PCA, CA and H_2O_2 were separated and coexisted in the equilibrium mixture. To our knowledge, this is the first example of a direct separation of peracid (e.g., PCA) from parent acid (e.g., CA) and H_2O_2 coexisting in the equilibrium mixture that is often analyzed with HPLC via derivatization method (20–22). The optimization and mechanism of the separation of CA, PCA, and H_2O_2 and their quantitative analyses are described later.

Effect of pH of the mobile phase

Figure 2 shows the effect of the mobile phase pH on the values of t_R of PCA and CA. No effect of pH on the t_R of H_2O_2 was noticed, indicating that H_2O_2 is not retained in the column used, though the t_R values of CA and PCA changed remarkably with pH. When the pH of the mobile phase was increased in the range of pH 2.2–5.5, the values of t_R of PCA and CA decreased, that is, the t_R values of PCA (CA) were 13.5 (14.9) and 12.0 (13.0) min at pHs of 2.5 and 2.9, respectively. The integrated areas of the well-separated peaks of CA and PCA (especially the chromatograms shown in Figures 2b and 2c) were found to increase as the mobile phase pH was increased. The ratio of peak areas for PCA and CA at pHs of 2.5 and 2.9 essentially remained constant. On the other hand, the peaks of PCA and CA were found to merge together, resulting in a single peak at pH ≤ 1.5 (Figure 2a), whereas a broad peak comprised three small peaks at the t_R range of 7.5–9.5 min was obtained at pH > 3.6 (Figures 2d and 2e). In addition, the so-called "fronting effect" (38) was observed at pH 2.9. When the chromatogram of CA was measured separately with the mobile phase at pH > 4 (the data is not shown), similar broadening of the peak was observed, that is, the single peak of CA was found to split into two diffused peaks at the t_R range of 8.0–10.0 min (discussed later).

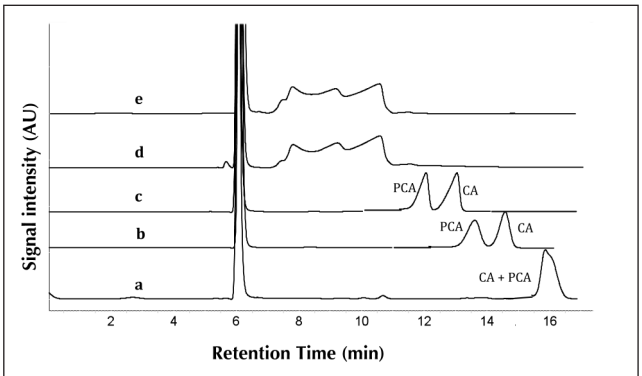

Figure 2. Chromatograms obtained for the reaction mixture containing PCA, CA and H_2O_2 with mobile phase of 3 mM $HClO_4$ at pHs: 2.2 (a), 2.5 (b), 2.9 (c), 4.0 (d), and 5.5 (e). Column temperature: 25°C.

Effect of $NaClO_4$

Figure 3 illustrates the typical chromatograms of the reaction mixture measured by varying the concentration of $NaClO_4$ additive in the mobile phase. Obviously, the selectivity of separation of PCA and CA was poor in the absence of $NaClO_4$ (Figure 3a), though it can be effectively improved by adding $NaClO_4$ additive in the eluting solvent (Figure 3b–3d). When the concentration of $NaClO_4$ was increased in the range of 13–450 mM, the t_R values of CA and PCA decreased and at the same time the separation between two peaks increased up to 33 mM (inset in Figure 3). In this case, no effect of $NaClO_4$ on the peak areas (or ratio) of CA and PCA were observed. Similar results were also obtained with other salts (e.g, sodium sulfate and sodium dihydrogen phosphate). Thus, the salt has a great effect on the present separation process (discussed later). In present study, 33 mM $NaClO_4$ was used as the additive in $HClO_4$ mobile phase for the analyses of PCA and CA.

Effect of column temperature

It is known that the temperature of the column has a considerable effect on the separation of ionic samples because it

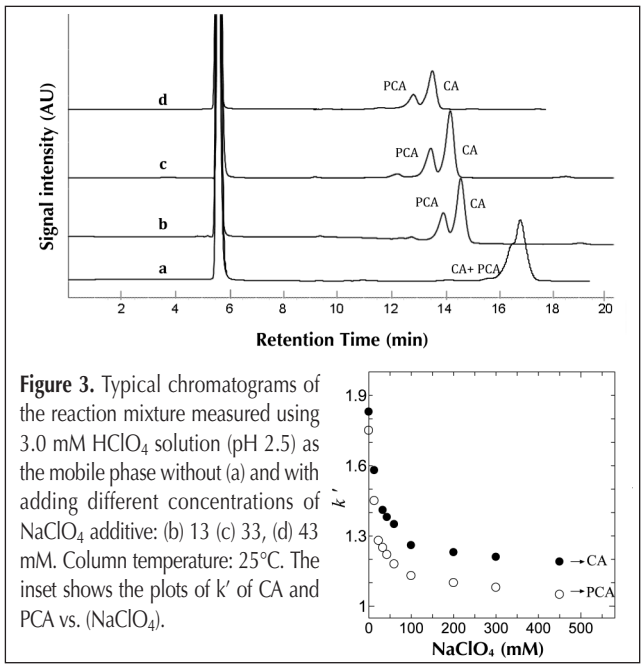

Figure 3. Typical chromatograms of the reaction mixture measured using 3.0 mM $HClO_4$ solution (pH 2.5) as the mobile phase without (a) and with adding different concentrations of $NaClO_4$ additive: (b) 13 (c) 33, (d) 43 mM. Column temperature: 25°C. The inset shows the plots of k′ of CA and PCA vs. ($NaClO_4$).

changes the degree of ionization and pKa of analyte, pH and viscosity (flow) of mobile phase, and the extent of interaction (adsorption) of analyte with stationary phase (39). Figure 4 shows the effect of column temperature on t_R and peak separation of PCA and CA coexisting in the reaction mixture. By increasing the temperature from 10 to 33°C, the t_R values of CA and PCA decreased (Figure 4), though the value of t_R of H_2O_2 remained unchanged. Also, an increase in column temperature led to a poor peak separation between PCA and CA (see the inset in Figure 4). The integrated areas of the chromatographic peaks for both PCA and CA were found to decrease with increasing column temperature up to 25°C, but increased again at 33°C. Moreover, the ratios of the peak areas of PCA and CA slightly decreased with increasing the temperature of column (the ratios were 0.54, 0.51, 0.49, and 0.54 at 10, 15, 25, and 33°C, respectively). The observed dependency of peak area on temperature may be associated with the change of absorption coefficient of the analyte. Therefore, the shorter t_R values of CA and PCA at the elevated temperature of column may be mainly associated with the temperature dependent degree of dissociation of analyte (i.e., the species of PCA and CA) and its interaction with the stationary phase. Moreover, the relatively poor separation observed at higher temperature was considered to result from the lower degree of adsorption of analyte with the column material. Although the best separation between CA and PCA could be achieved at 10°C, to avoid the cooling system of the column and to achieve a faster analysis (shorter t_R values), the present study was carried out at 25°C at which the separation is also satisfactorily established (inset in Figure 4).

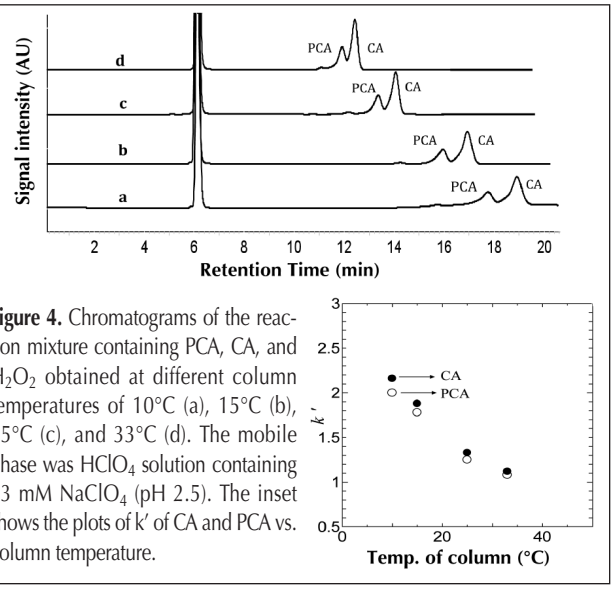

Figure 4. Chromatograms of the reaction mixture containing PCA, CA, and H_2O_2 obtained at different column temperatures of 10°C (a), 15°C (b), 25°C (c), and 33°C (d). The mobile phase was $HClO_4$ solution containing 33 mM $NaClO_4$ (pH 2.5). The inset shows the plots of k' of CA and PCA vs. column temperature.

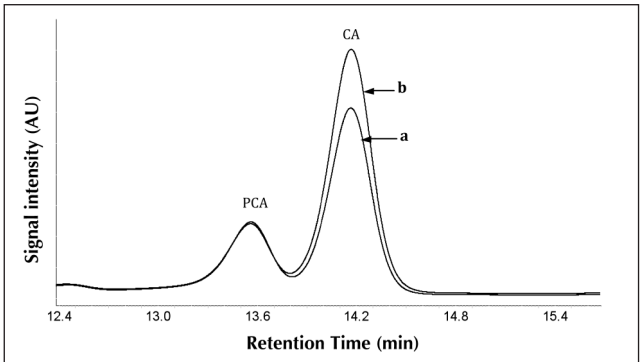

Figure 5. Typical chromatograms obtained for the reaction mixture containing PCA, CA and H_2O_2 before (a) and after (b) the standard addition of 2 mM of CA. The mobile phase was 3 mM $HClO_4$ solution containing 33 mM $NaClO_4$ (pH 2.5). Column temperature: 25°C.

Mechanism of the separation of PCA from CA

It was clarified previously that the eluting solvents containing acetonitrile and methanol result in a poor selectivity of separation between PCA and CA (Figure 1a and 1b). Because of a highly polar characteristic (i.e., low pKa values), PCA and CA were preferentially solvated by the water molecules, though they showed a little tendency for a solvation with acetonitrile and methanol. Thus, the observed poor selectivity of separation in the presence of acetonitrile and methanol in the mobile phase may be rational.

On the other hand, the separated responses for PCA and CA were successfully obtained in the presence of $NaClO_4$ in $HClO_4$ (Figure 3b–3d of Figure 3). Thus, it can be remarkably noted that $NaClO_4$ plays a vital role in the present separation process.

$$\text{Cit-COOH} \overset{K_{a1(CA)}}{\rightleftharpoons} \text{Cit-COO}^- + \text{H}^+ \quad p\mathit{K}a_1 = 3.1 \qquad \text{Eq. 2}$$

$$\text{PerCit-COOH} \overset{K_{a1(PCA)}}{\rightleftharpoons} \text{PerCit-COO}^- + \text{H}^+ \quad \text{pKa}_1 = 2.7 \qquad \text{Eq. 3}$$

The pKa values of –COOH groups in CA have been reported as 3.1, 4.6, and 6.4 (42), and it was recently used to determine two pKa values of PCA to be 2.7 and 4.5 using voltammetric and HPLC methods (11). It has been reported that the t_R value of the carboxylic acid [e.g., benzoic acid (34,39), salicylic acid (34), and succinic acid (39)] is increased with decreasing the mobile phase pH (34,35). Similarly, when the pH of the mobile phase was decreased, the t_R values of both PCA and CA increased. These pKa values of CA and PCA, except for pKa = 6.4, of CA was in the examined pH range of the mobile phase (2.2–5.5). Therefore, the observation of a broad chromatogram at pH 4.0 and 5.5 (Figure 2d and 2e) with shorter t_R value may be reasonably attributed to the dissociation of –COOH groups of CA and PCA as well as –COOOH group of PCA (Eqs. 2 and 3).

The close examination of the plots of k' vs. $NaClO_4$ for PCA and CA (inset in Figure 3) revealed that the extent of decrease in k' for PCA is slightly larger than that for CA. It is known that when the concentration of salt is increased up to a certain limit in the mobile phase, in general, the k' value of a neutral species increases, and it decreases for the charged one (34). Thus, the observed larger decrease in t_R of PCA with increasing the concentration of $NaClO_4$ (up to 40 mM) may be due to the increased electrostatic interaction (34) of the ions of $NaClO_4$ with the dissociated form of PCA (Eq. 3), resulting in a faster elution of PCA than CA.

Simultaneous analyses of PCA and CA

Selectivity and stability of PCA

Because PCA, CA, and H_2O_2 coexist in the equilibrium mixture (Eq. 1) and the difference between the t_R values of CA and PCA is

small (~ 0.5 min), prior to the analyses of PCA and CA, it would be required to verify the purity (i.e., selectivity of the column) of peaks of PCA and CA as well as the stability of PCA in the column. To justify these, a standard solution of CA was added in the reaction mixture containing known amounts of PCA and CA and the chromatograms were measured (Figure 5). It was noted that the addition of CA in the reaction mixture may negligibly increase the PCA concentration by the forward reaction shown by Eq. 1 within the measurement time, because the value of k' is significantly small (5.5×10^{-7} M^{-1} s^{-1}) (37). Furthermore, the decrease in PCA concentration by its hydrolysis (i.e., backward reaction of Eq. 1) after the separation of H_2O_2 in the column would be also taken as insignificant, because the value of k_{-1} is 2.5×10^{-7} M^{-1} s^{-1} (37). However, the addition of CA in the reaction mixture only increased the peak area corresponding to CA (note: the peak area was found to increase quantitatively with the added amount of CA), though the shape and area of the peak corresponding to PCA remained virtually unchanged. Previously, by studying the electrochemical and electrospray ionization mass spectroscopic analyses of PCA coexisting with CA and H_2O_2 (10), it was confirmed that the degree of backward reaction shown in Eq. 1 was negligibly small. Thus, present and previous observations essentially assure sufficient stability of PCA in the column and a reasonable degree of selectivity of the separation of PCA from the coexisting CA. Here, it should be unavoidably noted that the observed selectivity of the RP-HPLC separation of PCA from CA is appreciably sensitive to the presence of additive in the mobile phase, pH of the mobile phase and the temperature of the column used (discussed previously).

Intra- and inter-day precision and accuracy

The precision and accuracy of the developed method was validated and the obtained results are summarized in Tables I and II. The maximum intra-day precisions (i.e., C.V.) for CA and PCA were found to be 8.1% and 7.5%, respectively. The maximum inter-day precisions were 8.7% and 9.1% for CA and PCA, respectively. The inter- and intra-day accuracies for PCA were determined to be 93.22% and 95.21%, respectively, whereas those for CA were 90.23% and 95.21%, respectively.

Table I. Intra-day Precision and Accuracy of Analyses of PCA and CA Concentration

Conc. µM		C.V. (%)		Accuracy (%)	
CA	PCA	CA	PCA	CA	PCA
1.1	0.4	6.6	7.5	84.28	88.32
2.3	1.1	5.5	4.5	87.62	89.44
6.4	2.8	3.3	4.8	91.12	89.43
14.5	5.8	2.5	2.2	95.23	90.15
25.6	20.2	8.1	7.4	94.61	95.21

Table II. Inter-day Precision and Accuracy of Analyses of PCA and CA

Conc. µM		C.V. (%)		Accuracy (%)	
CA	PCA	CA	PCA	CA	PCA
1.1	0.4	8.7	9.1	80.43	79.33
2.3	1.1	6.8	5.5	85.35	86.14
14.5	5.8	5.7	4.8	90.21	93.22

Calibration curves of CA and PCA

In this case, 3.0 mM HClO4 solution (pH 2.5) containing 33 mM $NaClO_4$ was used as the mobile phase. Linearity for the calibration curves was achieved from 6.0×10^{-6} to 3.5×10^{-1} M for CA [the corresponding equation of the linear plot is as (peak area) = 6.838 × 109 (CA), r = 0.998] and 8.0×10^{-6} to 1.8×10^{-1} M for PCA [the corresponding equation of the linear plot is as (peak area) = 7.229 × 109 (PCA), r = 0.986]. The limits of quantification of CA and PCA were determined to be 1.12 µM and 0.43 µM, and the limits of detection of CA and PCA were determined to be 0.41 µM and 0.13 µM, respectively. The results demonstrated that without the so-called derivatization step (20–22), the optimized RP-HPLC method could be employed for the direct and simultaneous analyses of PCA, CA, and H_2O_2 coexisting in the equilibrium mixture. Such a direct analysis of PAA coexisting with AA and H_2O_2 in the equilibrium mixture has not been reported, although the separation of PAA using the post-column derivatization method has been carried out (20). This may be due to the less stability of PAA [note: the value of k^{-1} for PCA (2.5×10^{-7} M^{-1} s^{-1}) is by two orders of magnitude smaller than that (3.1×10^{-5} M^{-1} s^{-1}) of PAA (44)] in dilute solution (19,20,44).

Conclusions

The separation of PCA coexisting with CA and H_2O_2 in the equilibrium mixture, that allows the determination of molecular structure of PCA in their aqueous equilibrium mixture (10), could be successfully carried out using RP-HPLC method. It was found that the selectivity of the separation of PCA from CA and H_2O_2 is highly sensitive to the presence of additive in the mobile phase, pH of the mobile phase and the temperature of the column used. The conditions of separation were optimized by studying the effects of various additives in the mobile phase, pH of the mobile phase and the temperature of the column on the separation performance. The satisfactory separation of PCA from CA could be achieved with the mobile phase with a pH range of 2.2–2.9 at which PCA and CA are present mostly as dissociated and undissociated species, respectively. The linear calibration curves for PCA and CA were measured. This study significantly offers a fast, direct analysis of peroxyacids (e.g., PCA) that have been generally analyzed with RP-HPLC via the so-called pre- and post-column derivatization methods (18–20).

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