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# Application of micellar electrokinetic capillary chromatography for the determination of benzoic acid and its esters in liquid formula medicines as preservatives

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**Abstract**

We described a method for the simultaneous determination of preservatives including benzoic acid, methyl-, ethyl- and propyl-benzoate by micellar electrokinetic capillary chromatography (MECC). The factors affecting the reproducibility in the quantitative analysis of pharmaceuticals by MECC were investigated by varying the running buffer and washing condition inbetween runs. Preservatives in liquid formula medicines have been determined by optimum MECC condition using *p*-hydroxy benzoic acid as an internal standard. The reproducibility of this method was acceptable as a validate method for the quality control of pharmaceuticals (RSD < 2%). Routine quantitative analysis of pharmaceuticals using MECC could be possible with well characterized reproducible procedure. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Micellar electrokinetic capillary chromatography; Capillary electrophoresis; Quality control of pharmaceuticals; Benzoic acid and its esters; Preservatives; Quantitative analysis; Precision

## 1. Introduction

Capillary electrophoresis (CE) including micellar electrokinetic capillary chromatography (MECC) are perceived to be attractive tools for the analysis of pharmaceuticals because of their high separation efficiency, easy operation and low running cost. Quantitative analysis and the peak confirmation have to be possible for using CE as an analytical tool for quality control of pharmaceuticals. Determination of drugs and metabolites in body fluid were performed by CE and MECC [1–7] and the numerous studies presented recently [8–11]. These techniques provide valuable information from a qualitative point of view; however, they are only semiquantitative method. To prove CE method as a valid quantitative technique, further efforts have been attempted [12–14]. Reproducibility of migration time in CE is a key factor for valid quantitative analysis. The apparent mobility of an analyte is additive quantity of electrophoretic and electroosmotic mobility,

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which are affected by the capillary dimension, buffer concentration, the $\zeta$ potential of the silica surface, and the temperature of the system determining the viscosity, the dielectric constant and $\zeta$ potential. Using the buffer having an adequate buffering capacity and commercial instrument having stable capillary thermostating, variations of migration time are mainly due to the fluctuation of the electroosmotic mobility rather than that of electrophoretic mobility of analytes from run to run. It is difficult to control the electroosmotic mobility because of the dependence of electroosmotic mobility on the chemistry of the fused silica surface ($\zeta$ potential). $\zeta$ Potential of capillary surface can be varied depending on the history of capillary wall including washing procedure between runs, storage condition and age of capillary etc. Many efforts to obtain the reproducible surface have been done. First, the cleaning of the surface with NaOH between runs is the general method. Secondly, developments of reproducible and stable surface modification procedure for fused silica surface are progressing. Thirdly, the use of voltage preconditioning of capillary was reported [14]. Another approach for gaining the reproducible results was to use parameter of relative mobilities of the analytes and reference standard regardless of experimental condition [13]. In this study, preservatives including benzoic acid and methyl-, ethyl- and propyl-benzoate in liquid formula medicines were separated and quantitatively determined with MECC. Benzoic acid and its esters in food have been analyzed by reverse phase HPLC [15] and quantified by using the isotope dilution method [16]. For a more precise and accurate quantitative analysis with MECC, *p*-hydroxy benzoic acid was used as an internal standard. The factors affecting MECC separation and quantitative analysis of benzoic acid and its esters in liquid formula medicine were investigated. It turns out that the conditionings of capillary surface between runs were significant for the precision of retention time and peak area. Determinations of benzoic acid and benzoates in 28 real samples have been achieved.

## 2. Experimental

### *2.1. Materials*

Chemicals including benzoic acid (BA), *p*-hydroxybenzoic acid (PBA), methylbenzoate (methylparaben, MP), ethylbenzoate (ethylparaben, EP), propylbenzoate (propylparaben, PP), sodium phosphate and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO). Complex liquid formula medicines from various Korean pharmaceutical companies were obtained in local drug stores.

### *2.2. Capillary electrophoresis procedure*

For the analysis of the benzoic acid and its esters, the untreated fused silica capillary (Polymicro Technologies, Phoenix, AZ), 50 μm i.d. × 65 cm long (56.5 cm to detector) was used as a separation column. The apparatus used for these studies was an automated $HP^{3D}CE$ system (Hewlett Packard, Palo Alto, CA) with $HP^{3D}CE$ Chemstation for control and data acquisition. $HP^{3D}CE$ is equipped with a photodiode array UV detector, automatic pressure or electrokinetic sample injector, autosampler, peltier temperature controller, and 30 kV high voltage power supply. Prior to each run the capillary was rinsed with sodium hydroxide solution, distilled water and running buffer. The capillary was filled with running buffer, 20 mM sodium borate buffer, pH 7.8, containing 7.5 mM SDS unless specified. The samples were introduced by hydrodynamic injection (40 mbar for 10 s) and ran with the applying voltage of 30 kV at 25°C. For obtaining the reproducible run, the precondition of capillary was varied by flushing the capillary with pressure or applied voltage (indicated in the Figure legends). Samples were detected using a photodiode-array detector at 205 nm. Spectra were also collected during the runs for peak identification.

### *2.3. Sample preparations*

Standard stock solution of BA, PBA, MP, EP and PP were solubilized in triply distilled water by sonication, and a model mixture composed of

compounds was prepared by diluting the standard stock solution with triply distilled water to a concentration of 2–15 μg $ml^{-1}$. Real samples of preservatives in liquid formula medicine were obtained by diluting the sample ten-fold with doubly distilled water and removing the insoluble particles with centrifugation at 12000 rpm for 5 min. Quantitative analysis have been done by spiking

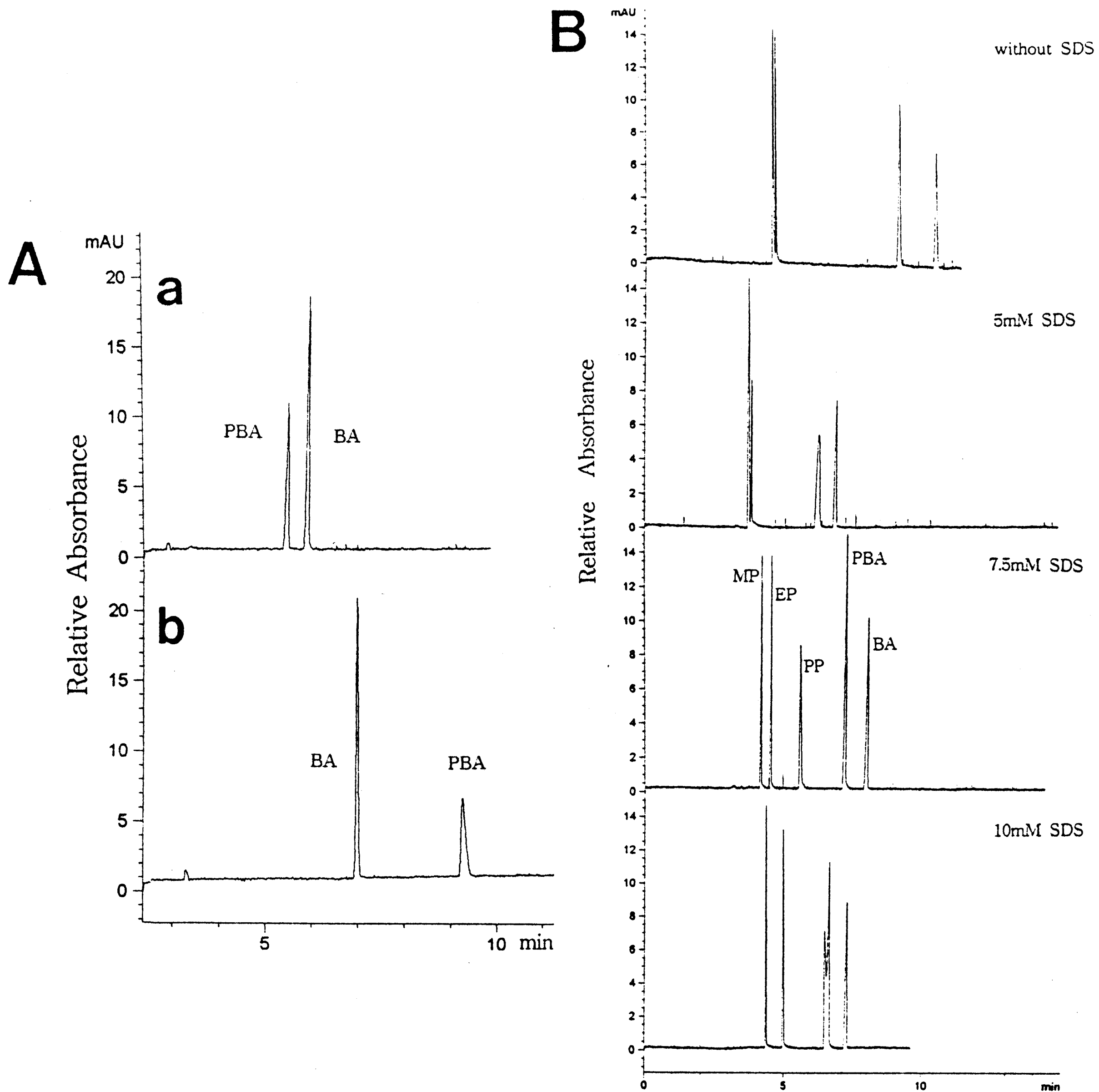


Fig. 1. Electropherogram of five benzoic acids and its ester (BA, PBA, MP, EP and PP) obtained from using: (A) 20 mM phosphate buffer without any additives at (a) pH 8.0 and (b) pH 9.6; (B) 20 mM phosphate buffer (pH 7.8) containing various concentration of SDS; 0 mM, 5, 7.5 and 10 mM SDS. Other CE conditions were followed: a fused silica capillary (50 μm i.d. × 65 cm long, 56.5 cm to detector); temperature, 25°C; applied voltage, 30 kV; injection, by pressure at 40 mbar for 20 s; UV detection wavelength, 205 nm.

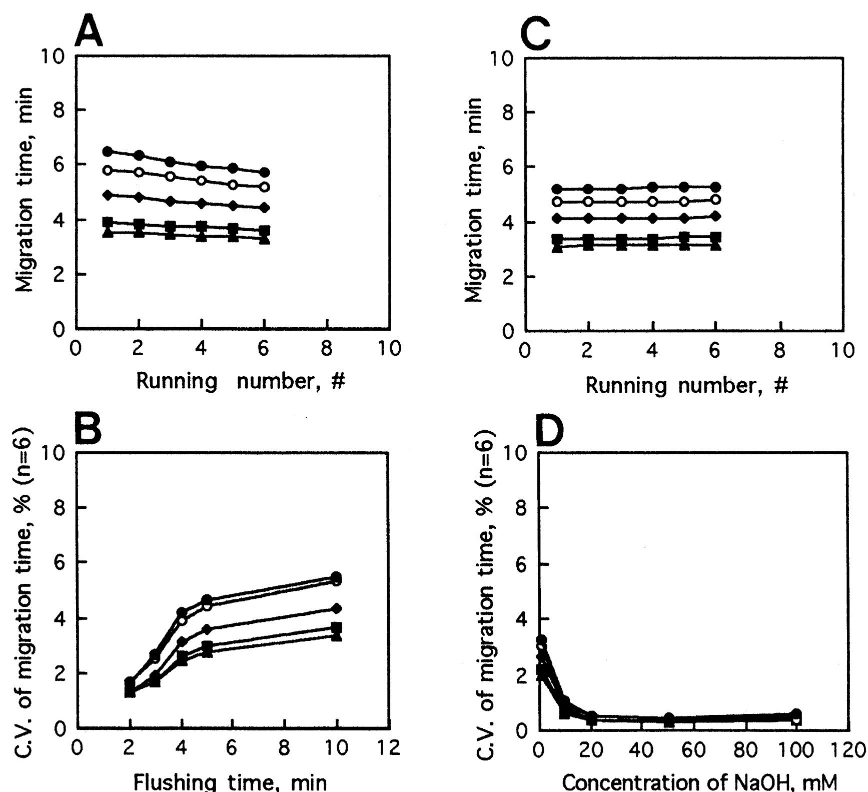


Fig. 2. Comparison of reproducibility depending on the washing procedure between runs. Variations of migration time of analytes were monitored (A,B) when the capillary was washed with only running buffer (20 mM phosphate buffer containing 7.5 mM SD, pH 7.8) for various times or (C, D) when the capillary was washed for 1.5 min with various concentrations of NaOH, 3 min with triply distilled water and 5 min with running buffer: (●), BA; (○), PBA; (▲), MP; (■), EP; (◆), PP.

the PBA in real sample to final concentration of 10 μg $ml^{-1}$ as an internal standard (I.S.).

## 3. Results and discussion

For the simultaneous determination of benzoic acid and its esters, the factors affecting the separation of benzoic acid (BA), p-hydroxy benzoic acid (PBA), methyl- (MP), ethyl- (EP) and propyl-paraben (PP) and reproducibility of migration time were examined. Because the error associated with the migration time of anionic compounds is affected to a greater degree than that of cationic species because of longer migration time, washing procedure to obtain the reproducible results was devised.

### *3.1. Optimization of the separation*

Optimum separations of anionic acids BA, PBA and neutral its esters MP, EP and PP with CE were achieved by varying the pH of the running buffer and the concentration of SDS in running buffer (20 mM sodium phosphate buffer, pH 7.8; Fig. 1). BA and PBA are negatively charged at pH 8.0, the electrophoretic mobility of more acidic BA ($pK_a = 4.58$) toward the anode is stronger than that of PBA ($pK_{a1} = 4.20$) and the migration time of PBA was shorter than that of BA (Fig. 1A-a). On the other hand, PBA mobility at pH 9.6 was reduced because the hydroxy group of PBA is deprotonated at $pK_{a2} = 9.31$ and doubly negative charged (Fig. 1A-b). The optimum pH of the running buffer was selected as 7.8, because electrophoretic mobility of anionic acids

toward the anode at pH 9.0 was too large for a quick separation and the band broadening was occured at pH 6.8 (data not shown). Without anionic detergent SDS, non-charged benzoates, MP, EP and PP could not be resolved as in Fig. 1B and the separations of these esters were improved by raising the SDS concentration up to 7.5 mM. If the SDS concentration in the running buffer was over 10 mM, the anionic PBA was overlapped with PP whose mobility was decreased because of the strong hydrophobic interaction with anionic SDS. The best separation of above five compounds was accomplished by using 20 mM sodium phosphate buffer (pH 7.8) containing 7.5 mM SDS. Each peak in electropherogram was confirmed by UV spectrum scanned with photodiode array detector (data not shown).

### *3.2. Effect of capillary preconditioning procedure on the reproducibility of migration time*

Because the variation of electroosmotic mobility is a main cause of poor reproducibility of CE, the capillary preconditioning is a critical procedure to gain the reliable reproducibility. Several preconditioning strategies were adopted; first, flushing the capillary with running buffer and applying the voltage at 10 kV for 5 min, secondly, flushing the capillary with running buffer for various times and thirdly, flushing the capillary with various concentrations of NaOH, triply distilled water and running buffer in order. The protocol preconditioning the capillary with 1 or 0.1 M of NaOH, distilled water and running buffer was commonly accepted. However, the fused silica surface of capillary is slowly etched and degenerated by washing with a high concentration of NaOH and the reproducibility of the used capillary column is diminished. We tried to wash the capillary with NaOH once at the end of the day, and flush the capillary with only running buffer at constant pressure or applied voltage for various times between runs. Preconditioning the capillary with only running buffer inbetween run was not good enough to obtain a better reproducibility. For example, when the capillary was flushed with running buffer at a constant pressure, the migration time of each analyte was consequently decreased as shown in Fig. 2A. As raising the flushing time with running buffer, migration time was more dramatically decreased and the reproducibilities of migration time in consequent six runs were decreased by increasing the flushing time with running buffer (C.V.s = 1.5–5.0%) as shown in Fig. 2B. This could be explained that the electroosmotic flow can be gradually increased by thickening the ionic double layer on the capillary surface with longer flushing time. Generally

Table 1
Reproducibility of migration time and peak area of benzoic acid and its esters using PBA as an internal standard

| Conc. (μg ml$^{-1}$) | Coefficient of variation (C.V. %) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Migration time | | | | Peak area | | | |
| | MP | EP | PP | BA | MP | EP | PP | BA |
| Within-run ($n = 5$) | | | | | | | | |
| 10 | 0.11 | 0.120 | 0.152 | 0.202 | 0.774 | 2.120 | 1.054 | 1.451 |
| 25 | 0.179 | 0.178 | 0.188 | 0.397 | 0.862 | 0.967 | 0.964 | 0.428 |
| 50 | 0.335 | 0.359 | 0.393 | 0.709 | 1.374 | 1.350 | 0.847 | 0.417 |
| 75 | 0.196 | 0.220 | 0.228 | 0.466 | 0.852 | 0.841 | 0.641 | 0.899 |
| Between-run ($n = 5$) | | | | | | | | |
| 10 | 0.393 | 0.415 | 0.504 | 0.677 | 1.158 | 1.570 | 1.957 | 0.330 |
| 25 | 0.496 | 0.523 | 0.664 | 0.836 | 1.680 | 1.832 | 1.709 | 0.235 |
| 50 | 0.521 | 0.557 | 0.686 | 0.924 | 1.243 | 1.783 | 0.794 | 0.586 |
| 75 | 0.577 | 0.652 | 0.906 | 0.893 | 0.575 | 0.316 | 0.542 | 0.394 |

CE conditions same as in Fig. 1 except running buffer (20 mM phosphate buffer containing 7.5 mM SDS, pH 7.8).

longer washing time with running buffer have been adopted in many laboratories for obtaining the fully equilibrated capillary surface. This study showed that the longer flushing time with running buffer could decrease the reproducibility of migration time. To remove the ionic double layer and initialize the capillary surface, sequential washing step with 0.1 ~ 1.0 M NaOH, triply distilled water and running buffer was employed for 1.5, 2 and 3 min, respectively. In this case, the migration time was slightly increased during repeated runs (Fig. 2C). This could occur because the electroosmotic flow is diminished by insufficient formation of ionic double layer after NaOH washing. This indicates that the reliable reproducibility of CE can be obtained with suitable combination of flushing time of NaOH, water and running buffer. Because there are too many possible combinations to accomplish the optimum reproducibility, we fixed the flushing time of NaOH (1.5 min), triply distilled water (3 min) and running buffer (3 min) and varied NaOH concentration from 0 to 100 mM. The reproducibility of migration time in sequential six runs is shown in Fig. 2D. Highest reproducibility has been obtained by flushing the capillary with NaOH ( > 20 mM) for 1.5 min, water for 3 min and running buffer for 3 min. It turned out that preconditioning the capillary surface and regulating the electroosmotic mobility were critical factors affecting the reproducibility of CE analysis as a validate method.

### 3.3. Analytical variables

#### 3.3.1. Precision

We assessed the precision of the method by repeated analyses of standard sample containing various concentrations of BA and its esters spiked with a known concentration of PBA as an internal standard (Table 1). The coefficients of variation (C.V.) of the retention time were less than 0.5% for within-run precision and less than 1.0% for the between-run precision. Those of the peak areas which obtained from the ratios of analytes to I.S., were less than 1.9% for both within- and between-runs.

#### 3.3.2. Detection limit

The detection limits for quantitative determination of BA and its esters were 0.05 ~ 0.2 μg ml$^{-1}$ through the whole procedure at signal-to-noise ratio, 3.

#### 3.3.3. Linearity

The calibration curves for BA and its esters are linear ( > 0.998 ~ 1.000) within 2 ~ 135 μg ml$^{-1}$, as shown in Fig. 3. The preservative contents in liquid formula medicines ranges between 60 ~ 1180 μg ml$^{-1}$ and the permissive limit of BA and its esters in food is 600 μg ml$^{-1}$. For the quantitative determination of BA and its esters in liquid medicines and food, real samples should be diluted ten-fold with triply distilled water.

#### 3.3.4. Recovery

Recovery of the sample was nearly 100% because there was no sample pretreatment.

### 3.4. Determination of BA and its esters in liquid formula medicines

Preservatives of BA and its esters in 28 of real liquid medicines containing cold medicines, digestants and tonic drinks have been quantitatively analyzed. Real samples spiked with a known amount of PBA (I.S.) were pretreated as described

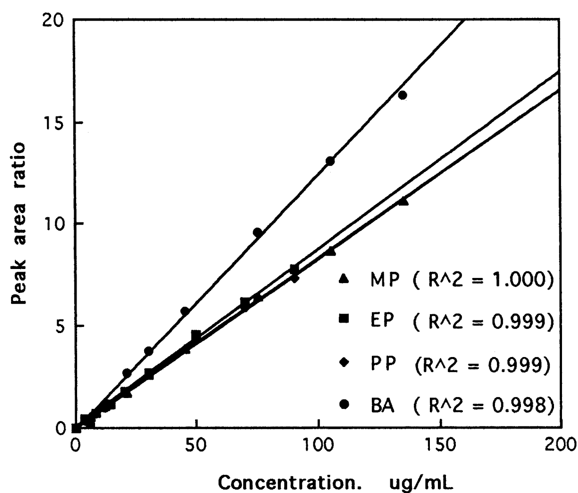


Fig. 3. Calibration curves for benzoic acid and its esters. (●), BA; (○), PBA; (▲), MP; (■), EP; (◆), PP.

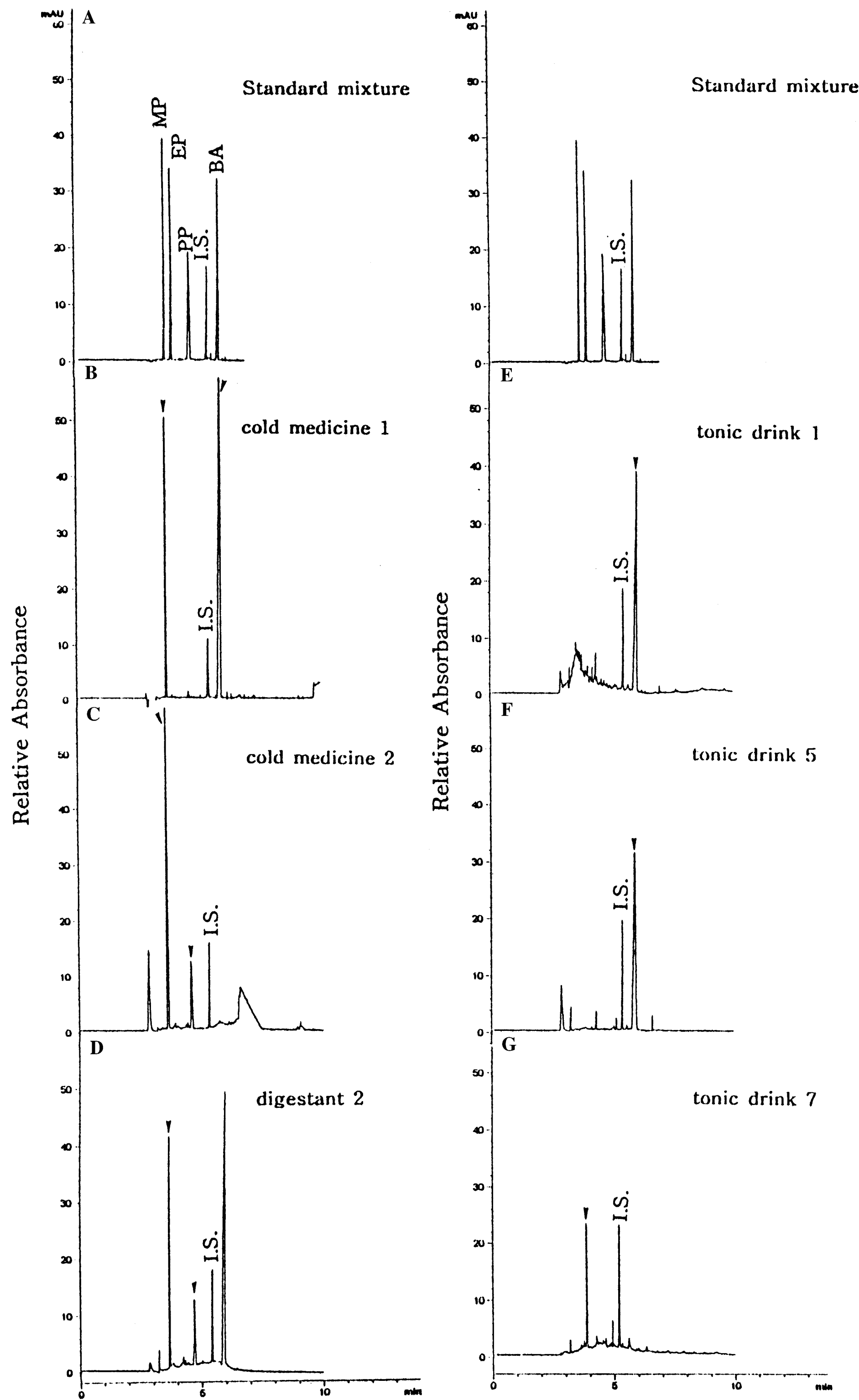


Fig. 4. Electropherograms obtained from (A) standard mixtures, (B, C) liquid formula cold medicines from different company, (D) liquid formula digestant, (E, F, G) tonic drinks from various companies after spiked with PBA as I.S. Arrow heads indicate the benzoic acid or its esters. The running conditions were the same as in Fig. 1C employing the same washing procedure as in Fig. 2C.

Table 2
The determination of the preservatives in various liquid formula medicines and its reproducibility

| Sample No. | Amount indicated on the label, μg ml$^{-1}$ | | | | Amount found mean, μg ml$^{-1}$ (n = 5, C.V., %) | | | | Found/indicated, % | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MP | EP | PP | BA | MP | EP | PP | BA | MP | EP | PP | BA |
| A. Cold medicine | | | | | | | | | | | | |
| 1 | 400 | | | 1000 | 382.3 (0.404) | | | 866.7 (0.322) | 95.6 | | | 86.7 |
| 2 | 800 | | 160 | | 648.0 (1.047) | | 143.5 (1.220) | | 81.0 | | 89.7 | |
| 3 | 800 | | 200 | | 694.5 (0.610) | | 187.5 (0.397) | | 86.8 | | 93.7 | |
| 4 | 500 | | 230 | | 391.1 (0.526) | | 216.6 (0.791) | | 78.2 | | 94.2 | |
| 5 | 900 | | 100 | | 605.6 (0.480) | | 70.2 (1.044) | | 67.3 | | 70.2 | |
| 6 | 700 | | 300 | | 487.0 (4.165) | | 217.0 (4.608) | | 69.6 | | 72.3 | |
| 7 | | | | 1180 | | | | 985.5 (0.416) | | | | 83.5 |
| 8 | 120 | | 60 | 950 | 109.3 (0.571) | | 56.2 (0.815) | 730.7 (0.535) | 91.1 | | 93.7 | 76.9 |
| 9 | | | | 1000 | | | | 789.3 (0.992) | | | | 78.9 |
| B. Digestant | | | | | | | | | | | | |
| 1 | | | | 120 | | | | 637.6 (0.241) | | | | 531.3 |
| 2 | 300 | | 150 | 650 | 271.0 (2.105) | | 146.0 (0.323) | 540.7 (0.349) | 90.3 | | 97.3 | 83.2 |
| 3 | 100 | | 40 | 1000 | 97.2 (0.578) | | 42.8 (0.603) | 850.1 (0.413) | 97.2 | | 107.1 | 85.0 |
| 4 | | 53 | | 1000 | | 40.9 (0.715) | | 725.9 (0.715) | | 77.2 | | 72.6 |
| 5 | 40 | | 20 | 800 | 44.3 (13.807) | | 20.1 (1.049) | 655.6 (0.318) | 110.8 | | 100.7 | 82.0 |
| C. Analeptic | | | | | | | | | | | | |
| 1 | | | | 600 | | | | 667.1 (0.234) | | | | 111.2 |
| 2 | | | | 600 | | | | 567.9 (0.312) | | | | 94.7 |
| 3 | | | | 700 | | | | 774.3 (0.673) | | | | 110.6 |
| 4 | 60 | | 40 | 700 | 79.0 (1.673) | | 65.9 (1.692) | 1050.4 (0.244) | 131.7 | | 164.7 | 150.0 |
| 5 | | 100 | 500 | | | 14.2 (1.200) | 584.4 (0.873) | | | 14.2 | 116.9 | |
| 6 | 70 | | 30 | 600 | 59.2 (0.659) | | 29.3 (2.102) | 436.9 (0.313) | 84.6 | | 97.7 | 72.8 |
| 7 | | | 100 | 600 | | | not found | 494.8 (0.507) | | | | 82.5 |
| D. Tonic drink | | | | | | | | | | | | |
| 1 | | | | ? | | | | 513.4 (0.822) | | | | |
| 2 | | | | ? | | | | 520.5 (0.091) | | | | |
| 3 | | | | ? | | | | 693.7 (0.194) | | | | |
| 4 | | | | ? | | | | 415.0 (0.563) | | | | |
| 5 | | | | ? | | | | 159.2 (0.467) | | | | |
| 6 | | | | ? | | | | 517.7 (0.284) | | | | |
| 7 | | ? | | | | 88.5 (0.966) | | | | | | |

in Experimental. Determinations of BA and its esters in various samples at optimized separation condition is shown in Fig. 4. To confirm the peak and to check the peak purity, UV spectra obtained from photodiode array detector have been compared with the standard spectra. No interference of other components in medicines were found. Quantitative analyses of BA and its esters in real samples were summarized in Table 2. The ratios of found to indicated amounts were $69 \sim 110\%$ and the low C.V.s which ranged from 0.1 to 2.1%, demonstrated the good precision of the method. The determined low values may come from the fluctuation of the real values, because benzoic acid derivatives as additives, not as active components are not strongly regulated in Korean FDA. The results show the possibility that the analytical procedure for the determination of BA and its esters in liquid medicines is acceptable as a validate method.

In this study, a simple and reproducible MECC method has been developed to determine BA and its esters simultaneously in liquid formula medicines. Optimum separation has been performed and the preconditioning procedure to obtain the reproducible results has been achieved. The developed method might be acceptable as a quantitative analytical method of pharmaceuticals.

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## References

[1] J. Caslavska, E. Gassmann, W. Thormann, J. Chromatogr. 709 (1995) 147–156.
[2] A. Schmutz, W. Thormann, Electrophoresis 15 (1994) 1295–1301.
[3] K.-J. Lee, J.J. Lee, D.C. Moon, Electrophoresis 15 (1994) 98–102.
[4] K.-J. Lee, G.S. Heo, N.J. Kim, D.C. Moon, J. Chromatogr. 608 (1992) 243–250.
[5] K.-J. Lee, J.J. Lee, D.C. Moon, J. Chromatogr. 616 (1993) 135–143.
[6] H. Nishi, S. Terabe, J. Chromatogr. 735 (1996) 3–27.
[7] D.K. Lloyd, J. Chromatogr. 735 (1996) 29–42.
[8] J. Luksa, D. Josic, J. Chromatogr. B 667 (1995) 321–327.
[9] S. Boonkerd, M. Lauwers, M.R. Detaevernier, Y. Michotte, J. Chromatogr. A 659 (1995) 97–102.
[10] P. Lukkari, T. Nyman, M.L. Riekkola, J. Chromatogr. A 674 (1994) 241–246.
[11] R.R. Chadwick, J.C. Hsieh, K.S. Resham, R.B. Nelson, J. Chromatogr. A 671 (1994) 403–410.
[12] H. Watzing, J. Chromatogr. 700 (1995) 1–7.
[13] C.P. Palmer, B.G.M. Vandeginste, J. Chromatogr. A 718 (1995) 153–165.
[14] G.A. Ross, J. Chromatogr. A 781 (1995) 444–447.
[15] Y. Ikai, H. Oka, N. Kawamura, M. Yamada, J. Chromatogr. 457 (1988) 333–343.
[16] W.J.S. Lockley, J. Chromatogr. 483 (1989) 413–418.