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Optimization of capillary liquid chromatography with electrochemical detection for determining femtogram levels of baicalin and baicalein on the basis of the FUMI theory

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ABSTRACT

Capillary liquid chromatography with electrochemical detection (CLC-ECD) was developed for determining the femtogram levels of baicalin and baicalein. The CLC-ECD system and the experimental conditions were optimized based on the precision (=information content) ϕ and efficiency (=information content/time) θ, which were calculated from a relative standard deviation (R.S.D.) by a chemometric tool called the FUMI theory. When CLC-ECD was established using a capillary column (Inertsil ODS-3, 150 mm × 0.2 mm i.d.), a sample injector fitted with a 0.2 μL injection loop, an applied potential of +650 mV *vs.* Ag/AgCl, and a flow rate at 1.8 μL/min, baicalin and baicalein were determined at femtogram levels. Moreover, the present method was validated using a chemometric tool and a conventional method. Since the FUMI theory makes it possible to predict R.S.D. without repetitive measurements, the chemometric tool saves considerable amounts of chemicals and experimental time, and was found to be useful for the optimization of conditions and validation for determination by CLC-ECD. The present method was applied to the analysis of Japanese Pharmacopoeia Scutellaria Root and *Scutellaria baicalensis* Georgi for determining baicalin and baicalein.

1. Introduction

High-performance liquid chromatography with electrochemical detection (HPLC-ECD) is considered to be a sensitive and selective method for determining redox compounds [1,2]. The downsizings of a column and flow line were reported to be effective for the improvement of sensitivity in HPLC-ECD [3–5]. Although capillary LC (CLC), which has a capillary column (0.1–0.5 mm inner diameter (i.d.)), has potential as a highly sensitive determination method, without suitable fabrication it may be less sensitive and have poor reproducibility. Thus, an optimization strategy for CLC systems and conditions is desired and essential to the development of highly sensitive CLC-ECD with extreme precision.

The operating conditions of HPLC have been refined to achieve high sensitivity, precision, and rapidity of analysis, and the miniaturization of the entire system has also been widely noticed to tackle economical and environmental issues [6–20]. The aim of this paper is to optimize a CLC-ECD system for determining baicalin and baicalein in Chinese herbal medicines.

This paper demonstrates an optimization method with precision and time as criteria. Precision is usually expressed as a standard deviation (S.D.) or a relative S.D. (R.S.D.) of measurements [21,22]. The plot of measurement R.S.D. against sample concentration, called precision profile [23–25], is a useful indicator to evaluate analytical methods. Practical use of the precision profile, however, poses a problem that an exact estimate of R.S.D. cannot be obtained without a large number of repetitions. To circumvent this problem, this paper adopts the FUMI (function of mutual information) theory [26,27] which can provide an R.S.D. estimate from the stochastic aspects of signal and noise in a chromatogram without repeating measurement [28,29].

In analytical chemistry, time is often incompatible with precision, *i.e.*, a precise method lacks time efficiency. This paper uses a chart which enables us to monitor precision and time using one chromatogram. Here, for convenience sake, precision is described as mutual information, ϕ(=log(1/R.S.D.)) and the analysis time, t, is replaced by efficiency, $\theta(=\phi/t)$ [30,31]. The precision and efficiency, ϕ and θ, can be calculated for each operating condition of an analytical system. Therefore, analytical performance can be represented as a point in the space spanned by ϕ on the X-axis and θ on the Y-axis. A continuous change in the condition (*e.g.*, methanol content in mobile phase) produces a trajectory in the ϕ–θ space, called ϕ–θ

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plot. We can identify the most precise condition and/or the most efficient condition from the ϕ–θ plot.

The methodology of this paper will give a suitable determination based on precision and efficiency and save energy, materials, and analysis time.

In addition, a strategy for judging the analytical performance of systems in a numerical way, called validation, has attracted attention, since measurements reported with any system need to meet an international standard [32–35]. The validation characteristics include accuracy, repeatability, intermediate precision, reproducibility, specificity, detection limit, quantitation limit, linearity, and range [21,35–37]. Another important factor, which is not among the validation characteristics, is time for analysis. If the FUMI theory is applied to the prediction of the measurement precision in a CLC-ECD without repeated measurements, chemicals and experimental time to examine validation characteristics will also be saved.

In this study, we were carried out the optimization and validation of CLC-ECD with high sensitivity for determining baicalin and baicalein in Chinese herbal medicines using the FUMI theory.

2. Experimental

2.1. Reagents

Baicalin (>99%) and baicalein (>98%) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other reagents and HPLC solvents (special HPLC grade) were from Wako Pure Chemical Industries, Ltd. Scutellaria Root of the Japanese Pharmacopoeia was obtained from Uchida Wakanyaku Co. Ltd. (Tokyo, Japan).

2.2. CLC-ECD system and conditions

The present CLC-ECD system consisted of a pump (MP-711i, GL Science, Tokyo), a sample injector (7520, Reodyne, Cotati, CA) fitted with a 0.5-μL or 0.2-μL or sample injector (C4-0004-05, Valco, Houston, TX) fitted with a 0.05 μL injection loop, a column, a mobile phase (phosphoric acid–methanol–water mixture (0.5:65:35, v/v/v)), and an electrochemical detector (LC-4C, BAS, Tokyo). The following columns were examined: a Capcell pak UG 120 ODS column (150 mm × 0.3 mm i.d., 3 μm, Shiseido, Tokyo), a Capcell pak MG 120 ODS column (150 mm × 0.3 mm i.d., 3 μm, Shiseido), an Inertsil ODS column (150 mm × 0.3 mm i.d., 3 μm, LC-Packing, Dionex, CA), an Inertsil ODS-3 column (150 mm × 0.3 mm i.d., 3 μm, GL Science), and an Inertsil ODS-3 column (150 mm × 0.2 mm i.d., 3 μm, GL Science). The column temperature was maintained at 40 °C. The electrochemical cell was constructed from a glassy carbon working electrode, an Ag/AgCl reference electrode, and a stainless steel counter electrode. The analog data of a chromatogram from the electrochemical detector were converted to digital chromatogram data by an A/D converter (24 bit, ChromatoMonitor, Nihon Filcon Co. Ltd., Tokyo); the digital data were recorded by a personal computer at sampling intervals of 0.4 s/point. The prediction of measurement R.S.D. based on the FUMI theory was performed with a software (MAY 2000, Hayashi Pure Chemical, Osaka, Japan).

2.3. Conventional HPLC–UV system and conditions

A conventional HPLC–UV was comprised of a pump (L-6000, Hitachi, Tokyo), a sample injector fitted with a 20 μL injection loop (model 7725, Reodyne), a Mightysil RP-18GP ODS column (150 mm × 4.6 mm i.d., 5 μm, Kanto Kagaku, Tokyo), and a UV detector (L-4000H, Hitachi). The baicalin and baicalein were also quantified by conventional HPLC according to the following conditions described in the Japanese Pharmacopoeia [37]: a mobile phase of a mixture of 0.6% phosphoric acid solution–acetonitrile (18:7, v/v), a flow rate of 0.8 mL/min, and the wavelength for detection of 277 nm.

2.4. Sample preparations

2.4.1. Scutellaria Root

To prepare a test solution, a sample of Japanese Pharmacopoeia Scutellaria Root (0.5 g) was added to 30 mL of 0.6% phosphoric acid solution–acetonitrile (18:7, v/v), heated under a reflux condenser in a water bath for 30 min, cooled, and filtered. The above procedure was repeated with the bark residue, using 30 mL of 0.6% phosphoric acid solution–acetonitrile (18:7, v/v) to make exactly 100 mL, and this solution was used as a test solution. The test solution was then passed through a 0.45 μm membrane filter. A 20-μL volume of the test solution was injected into a conventional HPLC–UV system. The test solution was diluted with a mixture of methanol–water–phosphoric acid (65:35:0.5, v/v/v) containing diethylstilbestrol (I.S.), and passed through a 0.45 μm membrane filter. A 0.2-μL volume of the dilute test solution was injected into the CLC-ECD system.

2.4.2. Scutellaria baicalensis Georgi

Pieces of bark, phloem, xylem, leaf, petiole, root, and the root bark of fresh *Scutellaria baicalensis* Georgi were chopped with a scissor or knife, and added to methanol containing diethylstilbestrol (I.S.) for the extraction of components. The methanol solutions were diluted with a methanol–water–phosphoric acid (65:35:0.5, v/v/v), and then passed through a 0.45 μm membrane filter. A 0.2-μL volume of the dilute test solution was injected into the CLC-ECD system.

3. Theory

Detailed explanations of the FUMI theory have been provided in papers by Hayashi and Matsuda [26,27]. In the FUMI theory, attention is paid to the phenomenological properties of noise. The time variation in the baseline of a chromatogram is described as the mixed random processes of white noise and a Markov process. White noise is a time-independent process with one parameter, but the Markov process has a time-correlation (also called the autocorrelation) with two parameters [38]. The digital noise data of 2 to the nth power points are transformed into the power spectrum by the Fourier transform and are least squares fitted for the parameterization of three parameters, the S.D., $\tilde{w}$, of the white noise and the S.D., $\tilde{m}$, and correlation parameter, ρ, of the Markov process. Finally, the noise parameters are used for the prediction of the measurement S.D. in the time space.

The following equation, $P(f)$, is the theoretical power spectrum of the mixed stochastic process of white noise and Markov process:

$$P(f) = \frac{\tilde{m}^2}{1-\rho^2} \times \frac{2\alpha}{\alpha^2 + 4\pi^2 f^2} + \tilde{w}^2 \quad (1)$$

where f denotes the frequency, $\alpha = (1-\rho)/\Delta t$, and Δt, are the sampling intervals for noise.

The chromatographic baseline is transformed into the power spectrum. The noise parameters of $\tilde{w}$, $\tilde{m}$, and ρ can be determined by the least squares fitting of Eq. (1) to the observed noise power spectrum. A power spectrum has the common natural phenomena feature where the power density at low frequencies is larger than that at high frequencies. It is often called $1/f$ fluctuation.

The noise parameters of $\tilde{w}$, $\tilde{m}$, and ρ are used for the estimation of the measurement R.S.D.: [26]

$$\text{R.S.D.}^2 = \frac{k_f \tilde{w}^2}{A^2} + \frac{\tilde{m}^2}{(1-\rho)^2 A^2}\left(k_f - 2\rho\frac{1-\rho^{k_f}}{1-\rho} + \rho^2\frac{1-\rho^{2k_f}}{1-\rho^2}\right) + I^2 \quad (2)$$

where k_f is the integration domain (peak width), A is the area of the signal parameter (peak area) and I is the R.S.D. of the volume error of the injector. The R.S.D. of the volume error of the injector, 0.003, is used, which is described in the specified form of the injector. At higher concentrations, peak area A becomes so large that the injection error is the most predominant factor in the precision. At lower concentrations, the contributions of the Markov process and white noise (the first and second terms of Eq. (2)) are much higher than the injection error. The utilization of this chemometric tool has provided measurement precision of various types of apparatuses in HPLC-ECD with conventional columns [28,29].

The mutual information ϕ_j for peak j takes the form: $\phi_j = -\log(S_j)$ (≥ 0) where S_j denotes the precision of measurements (the standard deviation of errors; the mean is unity) [30]. Let the total information ϕ be the sum of the individual information ϕ_j ($\phi = \sum_{j=1}^{q}\phi_j$). The information flow θ is defined as the time-average of the total information: [30]

$$\theta = \frac{\left(\sum_{j=1}^{q}\phi_j\right)}{t_q} \quad (3)$$

where t_q denotes the retention time of the last peak ($=\tau_q$) or the observation time (sufficient time to observe the whole shape of the last peak q) ($=\tau_q$ + constant). For a single-peak chromatogram, the flow θ can be given as $\theta = \phi_j/t_j$.

Two types of optimal conditions were proposed by Hayashi and Matsuda [31]. The ϕ-optimal was defined as the maximum of the total information ϕ among all the possible values of ϕ under the experimental conditions examined, corresponding to the most precise condition [39,40]. The θ-optimal is the most efficient (rapid) condition which takes the maximum of the information flow θ [40]. Hereafter, ϕ and θ are called precision and efficiency, respectively. Hayashi and Matsuda [31] examined the optimization of mobile phase composition, column length, flow rate (velocity), detection wavelength, and the amount of internal standard in HPLC–UV. Such optimization was referred to as total chromatographic optimization (TOCO).

For individual peak information, precision ϕ and efficiency θ are expressed as follows: [31]

$$\phi = \log\left(\frac{1}{\text{R.S.D.}}\right) \quad (4)$$

$$\theta = \frac{\phi}{t_R} \quad (5)$$

where R.S.D. means relative standard deviation of the peak area of peak j.

4. Results and discussion

4.1. Precision and efficiency in CLC-ECD predicted by the FUMI theory

To determine the three noise parameters: $\tilde{w}$, $\tilde{m}$, and ρ, 1024 digital data points (0.4 s/point) of the chromatographic baseline (Fig. 1A) were transferred to power spectra by Fourier transform (Fig. 1B, zigzag line). The noise parameters were determined as mentioned above, and the results are represented as the smooth line in Fig. 1B. With the parameters of $\tilde{w}$, $\tilde{m}$, and ρ and signal peak area over the integration domain, measurement R.S.D. can be estimated. The fit of the least squares in the FUMI theory indicates the predictability of the precision in our CLC-ECD system without repeated measurements (Fig. 1B).

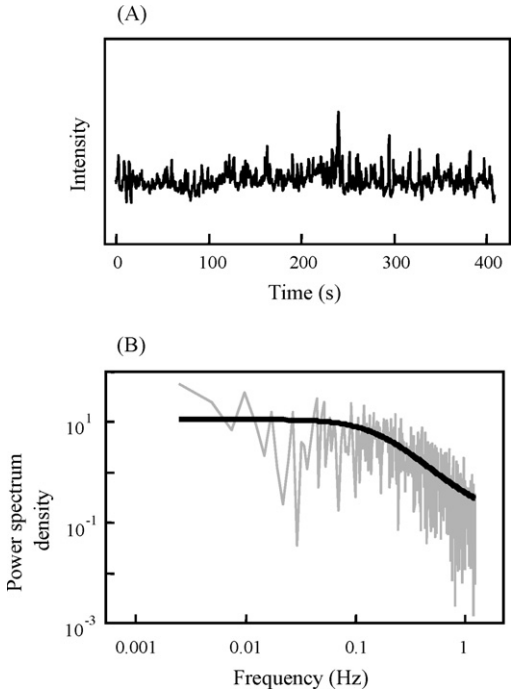

Fig. 1. Baseline of (A) chromatogram and (B) power spectra. Chromatographic baseline has been converted to power spectra by Fourier transform. The smooth solid line indicates the best fit of the theoretical line (white noise + Markov process) with the observed power spectrum (zigzag line). CLC conditions: mobile phase, phosphoric acid–methanol–water mixture (0.5:65:35, v/v/v); volume of sample injector, 0.2-μL; capillary column, Inertsil ODS-3 column (150 mm × 0.2 mm i.d., 3 μm); column temperature, 40 °C; applied potential, +650 mV *vs.* Ag/AgCl; flow rate, 1.8 μL/min.

Reverse-phase chromatography using an ODS column and a mobile phase of methanol–water mixture with 0.5% phosphoric acid was utilized for the determination of baicalin and baicalein. A chromatogram of baicalin and baicalein obtained by CLC-ECD is shown in Fig. 2A. In order to verify the usefulness of the FUMI theory for measurements in CLC-ECD, we compared the theoretical predicted R.S.D. with an experimentally observed R.S.D. Using five chromatograms, respectively, for 135, 67.6, 27.0, and 13.5 fg baicalein, the observed R.S.D. ($n = 5$) values are plotted *vs.* the amount of baicalein in Fig. 3. The predicted R.S.D. over a wide concentration range was obtained by the FUMI theory using only one chromatogram for 27.0 fg baicalein (solid line in Fig. 3). The predicted R.S.D. was identical to the observed R.S.D. When the chromatograms for 135, 67.6, and 13.5 fg baicalein were used to draw the precision plots for the determination of baicalein, respectively, the same curves, such as the solid line in Fig. 3, were also obtained from each chromatogram. In the case of baicalin, the predicted R.S.D. was also identical to the observed R.S.D.

This precision profile implies that the FUMI theory is useful for predicting the measurement precision of CLC-ECD without repeated measurements. While the experimental time to obtain the observed R.S.D. at four concentrations was 400 min (20 min × 5 time repeated measurement × four concentrations), the predicted R.S.D. required only 20 min. Thus, both chemicals and experimental time were saved using the FUMI theory.

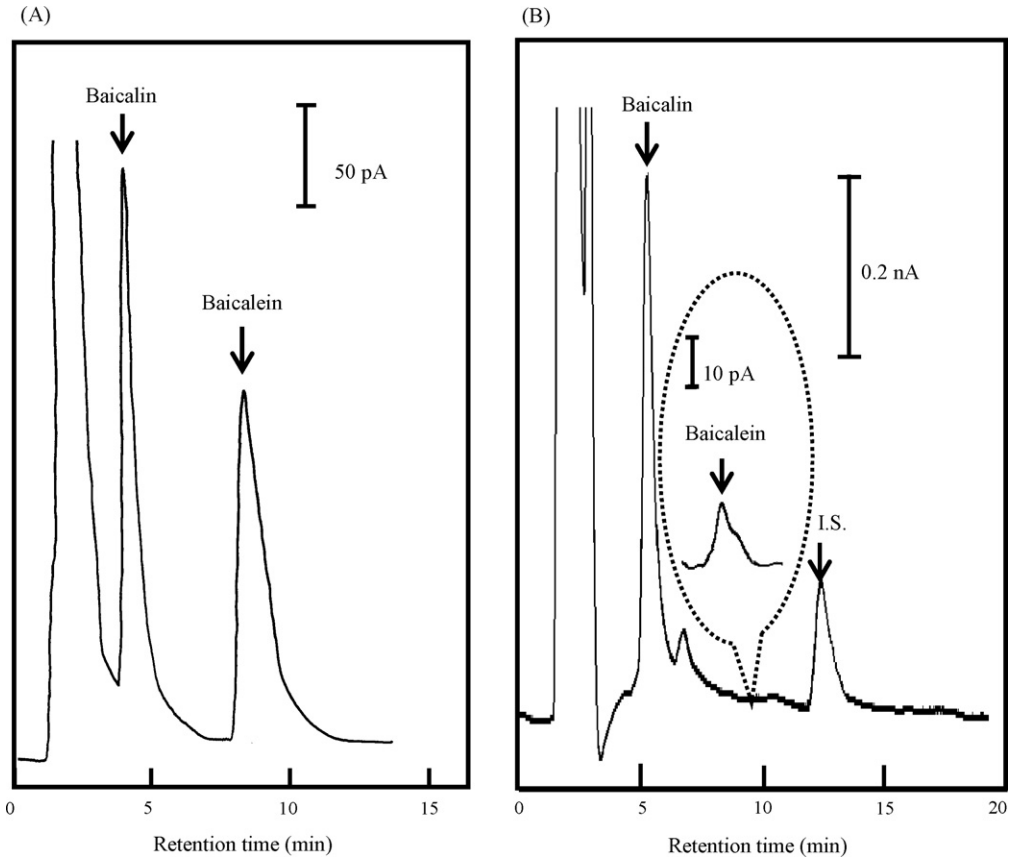

Fig. 2. Chromatograms of (A) standard of 892.7 fg baicalin and 540.5 fg baicalein and (B) extraction from a piece of Scutellaria Root of the Japanese Pharmacopoeia obtained by CLC-ECD. CLC conditions: mobile phase, phosphoric acid–methanol–water mixture (0.5:65:35, v/v/v); volume of sample injector, 0.2-μL; capillary column, Inertsil ODS-3 column (150 mm × 0.2 mm i.d., 3 μm); column temperature, 40 °C; applied potential, +650 mV *vs.* Ag/AgCl; flow rate, 1.8 μL/min.

4.2. Optimization of CLC-ECD using ϕ–θ plots

4.2.1. Selection of capillary columns

To select a capillary column with suitable separation and precision for determining baicalin and baicalein, five types of commercially available columns were tested: a Capcell pak UG 120 ODS column (150 mm × 0.3 mm i.d., 3 μm, Shiseido), a Capcell pak MG 120 ODS column (150 mm × 0.3 mm i.d., 3 μm, Shiseido), an Inertsil ODS column (150 mm × 0.3 mm i.d., 3 μm, LC-Packing, Dionex), an Inertsil ODS-3 column (150 mm × 0.3 mm i.d., 3 μm, GL Science), and an Inertsil ODS-3 column (150 mm × 0.2 mm i.d., 3 μm, GL Science). The baicalin and baicalein resolutions (Rs) using the columns examined, except for the Capcell pak UG 120, were more than 6.54 (Table 1). In this study, the ϕ and θ values of baicalin and baicalein were obtained from the chromatograms of 892.7 fg baicalin and 540.5 fg baicalein. The precision ϕ and the efficiency θ of baicalein were calculated for each column at 1.4 μL/min, and these results have been listed in Table 1. The largest ϕ was in CLC-ECD using an Inertsil ODS-3 column (150 mm × 0.2 mm i.d., 3 μm), thereby this capillary column was selected as the best one. Although the large θ of baicalein was in CLC-ECD using a Capcell pak UG 120, baicalin's peak overlapped the solvent peak on the chromatogram because the partition of baicalin for the stationary phase would be weak. In

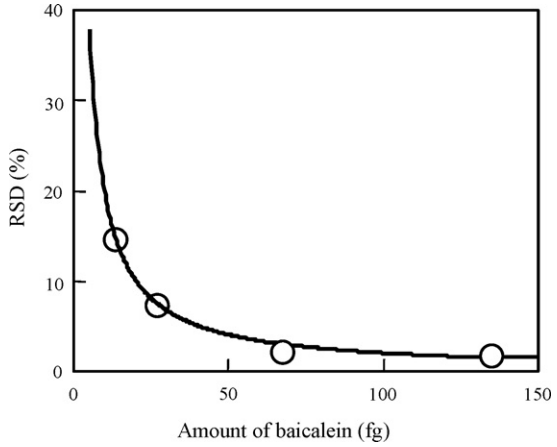

Fig. 3. Precision plots for the determination of baicalein using CLC-ECD. Observed R.S.D.s calculated from chromatographic peaks of baicalein are indicated by open circles. The repetition number of measurements (n) is five at each amount. Although the solid lines mean predicted R.S.D. based on the FUMI theory using a chromatographic peak for 135, 67.6, 27.0 and 13.5 fg baicalein, respectively, each curve using a chromatographic peak at each concentration overlapped.

Table 1
Precision ϕ and efficiency θ of baicalein and resolution (Rs) of baicalin and baicalein by CLC-ECD using various capillary columns

Column (manufacturer)	i.d. (mm)	ϕ	θ	Rs
Capcell pak UG 120 (Shiseido)	0.3	2.15	0.16	×[a]
Capcell pak MG 120 (Shiseido)	0.3	1.97	0.11	9.83
Inertsil ODS-3 (LC-Packing)	0.3	1.69	0.12	7.63
Inertsil ODS-3 (GL Science)	0.3	1.92	0.13	6.54
Inertsil ODS-3 (GL Science)	0.2	2.18	0.12	6.75

The length and ODS particle size of each column was 150 mm and 3 μm, respectively.
[a] Rs of baicalin and baicalein could not be obtained because baicalin's peak overlapped with the solvent peak on the chromatogram.

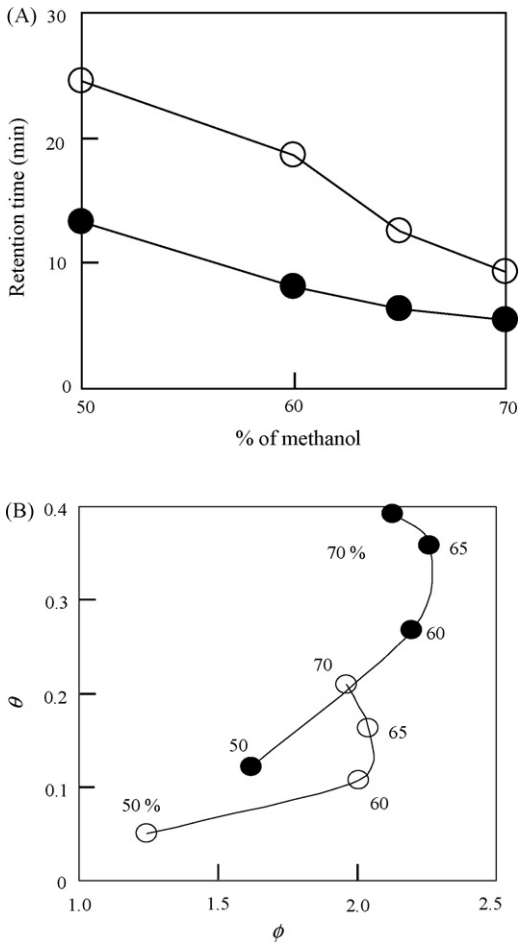

Fig. 4. Effect of methanol percentage on mobile phase on (A) retention time and (B) the precision ϕ *vs.* the efficiency θ of (●) baicalin and (○) baicalein. The methanol percentage in mobile phase of each measurement is indicated near the plot in the figure.

any circumstance of optimization experiments, a sufficient resolution (Rs > 1.5) should be ensured and under such as a condition, the ϕ–θ plot will be available. Table 1 lists the examples of the calculated precision and efficiency with good resolution, but the Shiseido column (Capcell pakUG120) is an exception. Since the θ of the column was exceptional, it is listed as a matter of reference.

4.2.2. Determination of mobile phase composition

The effect of the methanol ratio on the mobile phase is shown in Fig. 4. The retention time became smaller with the increase in methanol (Fig. 4A). The precision ϕ and efficiency θ calculated are plotted under the different percentages of methanol in the mobile phase, called ϕ–θ plots, and shown in Fig. 4B. The most precise analysis and the highest throughput analysis, can be indicated by the maximal values of ϕ and θ, respectively. As can be seen in Fig. 4B, the methanol content in the mobile phase shows its own trajectory pattern in the ϕ–θ plot: here, the u-shaped trajectory. We can see that the most precise condition is 65% methanol and the most efficient is 70%. In this study, a mixture of methanol–water (65:35, v/v) was chosen as the most suitable mobile phase since it gave priority to the most precise condition.

4.2.3. Selection of sample injector volume and optimization of flow rate

To select a suitable sample injector volume with a high precision for determining baicalin and baicalein, 0.05, 0.2, and 0.5 μL sample injector volumes were examined. In Fig. 5, ϕ values were plotted against θ values for each sample injector volume and flow rate. The capillary column pressure was kept under 15 MPa at the flow rates. CLC-ECD with a 0.2 μL sample injector volume at more than 1.4 μL/min flow rate gave relatively large ϕ values. Because the maximal values of ϕ and relatively large θ were obtained under CLC-ECD conditions using a 0.2 μL sample injector volume at a flow rate of 1.8 μL/min, these conditions were selected as optimal.

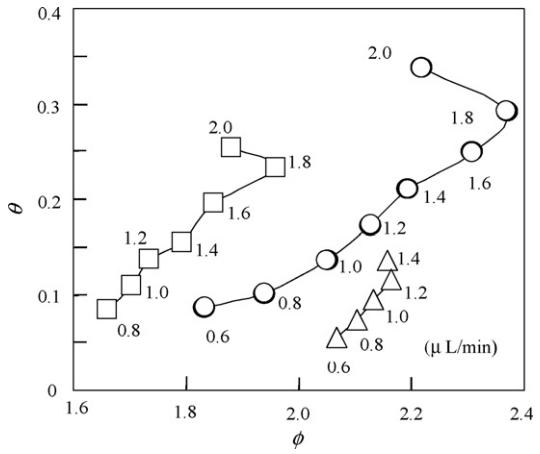

Fig. 5. Effect of flow rate on the precision ϕ *vs.* the efficiency θ obtained using a sample injector with various volumes at each flow rate. Volume of the sample injector: (△) 0.5 μL; (○) 0.2 μL; (□) 0.05 μL. The flow rate of each measurement is indicated near the plot in the figure.

4.2.4. Optimization of applied potential

The optimization of the applied potential of the detector was studied for baicalin and baicalein in CLC-ECD. Hydrodynamic voltammograms for 892.7 fg baicalin and 540.5 fg baicalein are shown in Fig. 6A. Although peak height was the greatest at +900 mV *vs.* Ag/AgCl, chromatographic baseline noise is large and the reproducibility of the peak height was poor at this potential. As described above, the predicted R.S.D. of baicalin and baicalein can be obtained directly from only one chromatogram according to the FUMI theory. The precision ϕ and efficiency θ obtained were plotted against the detection potential, called ϕ–θ plots and are shown in Fig. 6B. The chromatographic noise and signal, that is to say the precision ϕ, were affected by the applied potentials, and the ϕ–θ plot thus became a straight line. The largest ϕ for baicalin and baicalein determination were at +650 mV and +900 mV *vs.* Ag/AgCl, respectively. In the case of the simultaneous determination of baicalin and baicalein in a sample containing complex matrices such as Chinese herbal medicines, the applied potential in the presented CLC-ECD was selected at +650 mV *vs.* Ag/AgCl.

Thus, the optimal CLC-ECD for conditions determining baicalin and baicalein in this study were: capillary column, Inertsil ODS-3 column (150 mm × 0.2 mm i.d., 3 μm); volume of sample injector, 0.2 μL; mobile phase, methanol–water–phosphoric acid (65:35:0.5, v/v/v); flow rate, 1.8 μL/min; and applied potential, +650 mV *vs.* Ag/AgCl.

4.3. Method validation of CLC-ECD

4.3.1. Detection and quantitation limits

As mentioned above, the FUMI theory can estimate the R.S.D. of measurements from the probabilistic properties of noise and signal in a chromatogram without recourse to usual repetition. Given a calibration line, the FUMI theory can provide both detection and quantification limits. Here, the detection limit, L_D, is defined

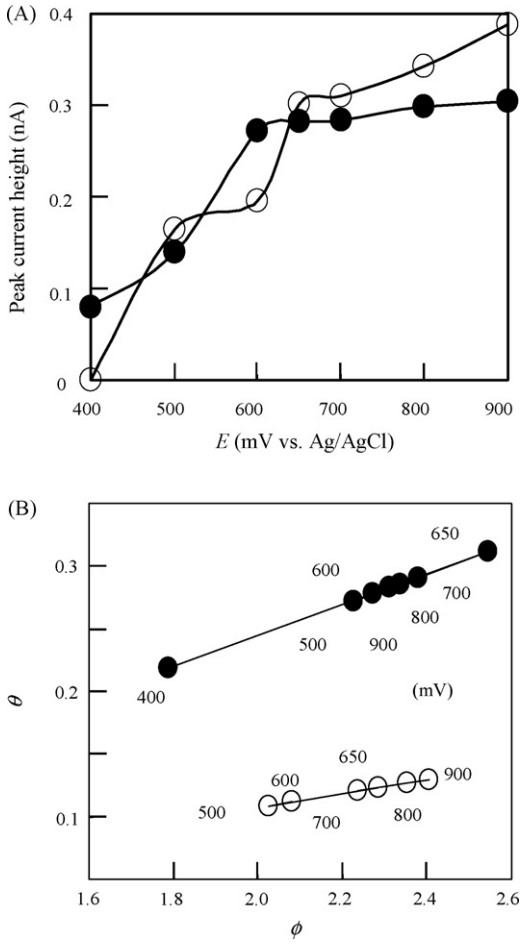

Fig. 6. (A) Hydrodynamic voltammogram and (B) relationship precision ϕ and efficiency θ of baicalin (●) and baicalein (○) at each applied potential.

as $L_D = 3s/a$ where s means the S.D. of measurement (=σ of FUMI theory) and a implies the slope of the calibration line [41]. We can notice from the equation that $(s/a)/L_D = 1/3 = 33.3\%$, the detection limit is the concentration, L_D, if the R.S.D. of measurements, $(s/a)/L_D$, shows 33.3%. We should note that s/a means the S.D. with the dimension of concentration and then $(s/a)/L_D$ denotes the R.S.D. with respect to concentration. Since the confidence interval of the detection limit by the FUMI theory is ±20%, which is equal to S.D. error from 40 repetitive measurements [42], a detection limit based on the FUMI theory has reliability. The detection limits of baicalin and baicalein were 9.82 and 7.03 fg, respectively. Usually a detection limit in chromatographic methods is obtained using a signal-to-noise ratio (S/N = 3). The detection limits (S/N = 3) of baicalin and baicalein by the present CLC-ECD were 9.76 and 7.14 fg, respectively. Thus, the detection limits of baicalin and baicalein by the FUMI theory and S/N were essentially the same. The sensitivity was compared with other available methods for determining baicalein. The detection limits of baicalein by μHPLC-ECD [5] using a microbore ODS column (150 mm × 1.0 mm i.d.), HPLC–UV [43] using an ODS column (250 mm × 4.6 mm i.d.), HPLC-ECD [44] using an ODS column (250 mm × 4.6 mm i.d.), and LC-MS [45] using an ODS column (200 mm × 4.6 mm i.d.) were 540 fg, 17.6 ng, 108 pg, and 129 pg, respectively. The present CLC-ECD is the most sensitive technique among the above common methods.

The definition of the quantification limit L_Q, is defined as $L_Q = 10s/a$ [36,37]. In the same manner, we can see that the quantification limit is the concentration when the measurement R.S.D. is 10%. The quantitation limits of baicalin and baicalein were 32.7 and 23.4 fg, respectively. Usually a quantitation limit in chromatographic methods is obtained using a signal-to-noise ratio (S/N = 10). The quantitation limits (S/N = 10) of baicalin and baicalein by the present CLC-ECD were 32.5 and 23.8 fg, respectively. Thus, the quantitation limit of baicalin and baicalein by the FUMI theory and S/N were also essentially the same.

4.3.2. Accuracy, linearity, and repeatability

The accuracy of the calculated data, as relative error (RE) at 2232, 892.7, 446.4, 223.2, 111.6, and 44.64 fg baicalin and 1351, 540.5, 270.2, 135.1, 67.56, and 27.02 fg baicalein, were within the range of −1.03 to 2.16% and −2.7 to 2.8%, respectively, as shown in Table 2. It was noted that the present CLC-ECD method provides quite accurate measurements of baicalin and baicalein.

Peak area was found to be linearly related to the amount of baicalin and baicalein from 32.7 fg to 2.23 pg ($r > 0.998$) and 23.4 fg to 1.35 pg ($r > 0.998$), respectively, *i.e.*, the concentration of baicalin and baicalein in the standard solution from 164 to 11.2 ng/mL ($r > 0.998$) and 117 to 6.75 ng/mL ($r > 0.998$), respectively. In addition, the regression equation with regression coefficients showed good linearity.

Although accuracy and linear range were evaluated by actual chromatogram measurement for each amount, repeatability as R.S.D. was obtained using the FUMI theory. The R.S.D.s of the chromatographic peak areas of baicalin and baicalein at each amount are listed in Table 2. In the case of the evaluation of the intermediate precision or reproducibility, actual chromatogram measurements of each condition are necessary. However, because the FUMI theory can provide an R.S.D. estimate from the stochastic aspects of signal and noise in a chromatogram without repeating measurement, processing time to obtain an R.S.D. of intermediate precision or reproducibility can be decreased.

4.3.3. Robustness

Moreover, the robustness for applied potential and the flow rate in the present CLC-ECD for determining baicalin and baicalein were evaluated. The effect of small changes in the applied potential (+650 mV *vs.* Ag/AgCl) and the flow rate (1.8 μL min) on R.S.D. of peak area and retention time of baicalin and baicalein were examined. These results are summarized in Table 3. The effects of the applied potential and flow rate in the examined conditions were found to be minor in terms of precision and separation. Moreover, the effect of 5% changes in methanol percentage in the mobile phase on peak areas and retention times of baicalin and baicalein were also examined. Although the R.S.D. of the retention time was obtained by repetitive measurements of the chromatograms, detection limit, quantitation limit, repeatability, and robustness against peak area were easily evaluated using the FUMI theory.

4.4. Distribution of baicalin and baicalein in Scutellaria baicalensis Georgi

Baicalin and baicalein in Japanese Pharmacopoeia Scutellaria Root were determined by the present CLC-ECD method. A typical chromatogram for Scutellaria Root is shown in Fig. 2B. Baicalin and baicalein contents in the Scutellaria Root are listed with their recovery data in Table 4. The R.S.D.s for baicalin and baicalein were less than 0.66% ($n = 5$). Baicalin's and baicalein's recoveries for spiked test solutions were more than 98.8% and their R.S.D.s were less than 0.73% ($n = 5$). By comparing the analytical results obtained by CLC-ECD and conventional HPLC–UV methods [37] (Table 4), it was noted that both results of baicalin were essentially identical. In the Japanese Pharmacopoeia, the content of baicalin in Scutellaria Root is regulated to be not less than 10.0%, calculated on the basis of dried

Table 2
Accuracy and repeatability for the measurement of baicalin and baicalein peaks

Baicalin				Baicalein			
Nominal amount (fg)	Determined amount (fg)	Accuracy (%, RE)	Repeatability[a] (%, R.S.D.)	Nominal amount (fg)	Determined amount (fg)	Accuracy (%, RE)	Repeatability[a] (%, R.S.D.)
2232	2273	1.84	0.17	1351	1380	2.2	0.19
892.7	903.0	1.16	0.43	540.5	525.9	−2.7	0.45
446.4	450.8	0.97	0.81	270.2	272.4	0.8	0.85
223.2	220.9	−1.03	1.55	135.1	139.6	3.3	1.72
111.6	110.7	−0.85	2.71	67.56	65.88	−2.5	2.90
44.64	45.62	2.16	6.05	27.02	27.78	2.8	7.37

[a] R.S.D.s were calculated by the FUMI theory.

Table 3
Evaluation of robustness for (A) applied potential, (B) flow rate, and (C) % of methanol in mobile phase against chromatographic peak area and retention time

A

Applied potential (mV *vs.* Ag/AgCl)	Baicalin		Baicalein	
	Peak area ratio	R.S.D.[a]	Peak area ratio[a]	R.S.D.[a]
648	0.992	0.43	0.978	0.45
650	1	0.43	1	0.45
652	1.01	0.42	1.03	0.40

B

Flow rate (μL/min)	Baicalin				Baicalein			
	Peak area		Retention time		Peak area		Retention time	
	Peak area ratio	R.S.D.[a]	Retention time ratio	R.S.D.[b]	Peak area ratio	R.S.D.[a]	Retention time ratio	R.S.D.[b]
1.7	0.939	0.43	1.05	1.29	0.895	0.50	1.04	1.00
1.8	1	0.43	1	1.28	1	0.45	1	0.99
1.9	1.01	0.42	0.938	1.27	1.03	0.44	0.953	1.08

C

% of methanol	Baicalin				Baicalein			
	Peak area		Retention time		Peak area		Retention time	
	Peak area ratio	R.S.D.[a]	Retention time ratio	R.S.D.[b]	Peak area ratio	R.S.D.[a]	Retention time ratio	R.S.D.[b]
60	0.952	0.48	1.29	1.29	0.971	0.48	1.39	0.99
65	1	0.43	1	1.28	1	0.45	1	0.99
70	0.893	0.57	0.86	1.24	0.953	0.50	0.74	1.03

[a] R.S.D.s of peak area were calculated by the FUMI theory.
[b] R.S.D.s of retention time were obtained from repetitive measurements ($n = 3$).

Table 4
Contents of baicalin and baicalein in Japanese Pharmacopoeia Scutellaria Root and recovery from Scutellaria Root spiked with baicalin and baicalein standards by CLC-ECD and HPLC–UV methods

Method	Content[a] ($n = 5$)		Recovery[b] ($n = 5$)		
	Amount (mg/g)	R.S.D. (%)	Added amount (mg/g)	Recovery (%)	R.S.D. (%)
CLC-ECD					
Baicalin	145	0.66	150	98.8	0.73
Baicalein	2.28	0.57	2.0	99.6	0.61
HPLC–UV					
Baicalin	148	1.87	150	98.7	1.79
Baicalein	N.D.	–	150	99.1	1.75

[a] Baicalin and baicalein amounts in Scutellaria Root were first determined using the sample preparation procedure in Section 2.4.1.
[b] For the recovery test, baicalin and baicalein standards at each amount were directly spiked to Scutellaria Root. Baicalin and baicalein derived from both Scutellaria Root and the standard were extracted, and a test solution was injected into CLC-ECD to obtain a chromatogram. The recovery was 100% when the baicalin or baicalein amount in the recovery test was equal to the sum of baicalin or baicalein in Scutellaria Root and the spiked standard.

material. In this study, although baicalein in Scutellaria Root could not be detected by conventional HPLC–UV with the above sample preparation, the present CLC-ECD method was able to determine baicalein content.

The distribution of baicalin and baicalein in *S. baicalensis* Georgi was also analyzed by the present method. The content (±S.D., $n = 6$) of baicalin in leaf, petiole, bark, phloem, xylem, root bark, and root was 87.4 ± 1.75, 8.75 ± 0.25, 33.4 ± 0.5, 43.5 ± 0.96, 18.7 ± 0.47, 291 ± 5.53, and 1677 ± 21.8 μg/g, respectively. The content (±S.D., $n = 6$) of baicalein in leaf, petiole, bark, phloem, xylem, root bark, and root was 0.753 ± 0.02, 0.784 ± 0.02, 0.410 ± 0.01, 0.317 ± 0.007, 0.260 ± 0.005, 2.45 ± 0.05, and 13.5 ± 0.32 μg/g, respectively. A root of *S. baicalensis* Georgi, from which the periderm had been removed, was used to make a Scutellaria Root. Therefore, the baicalin and baicalein content in the root of *S. baicalensis* Georgi were most abundant among the other positions. The results demonstrate that the present CLC-ECD method provides sensitive and quite accurate measurements of baicalin and baicalein in *S. baicalensis* Georgi.

5. Conclusions

An optimization strategy using the FUMI theory has been applied to develop a CLC-ECD system for determining femtogram

levels of baicalin and baicalein. Since the FUMI theory makes it possible to predict R.S.D. in CLC-ECD from only one chromatogram with no requirement for repetitive chromatographic measurements, the use of the FUMI theory helped save considerable amounts of chemicals and experimental time, and was found to be useful for the optimization of conditions and validation for the determination by CLC-ECD.

Precision, ϕ, and efficiency, θ, are the parameters to evaluate the analytical performance of a given method in a numerical way. The most advantageous point of this optimization design is that the most precise method and/or the most efficient method can be selected among a variety of experimental conditions. By definition, ϕ and θ are dependent, and they might seem to provide almost the same or close results of optimization. In practice, however, the condition with the highest ϕ does not necessarily coincide with that of the highest θ (see Figs. 4–6). This is partly because the time for analysis is independent of ϕ or θ. Every experimental condition has the values of phi and theta of its own and can be represented as a point in the ϕ–θ plot. Moreover, the continuous change in the condition (*e.g.*, MeOH content in mobile phase, wavelength, and column length) leads to a trajectory in the ϕ–θ plot. The conditions can be characterized by the different patterns of the trajectory as shown in Figs. 4–6. From the ϕ–θ plot, we can see not only the optimum conditions but also the effects of the changes in the conditions on analytical performance. Two parameters: precision ϕ and efficiency θ, were calculated from R.S.D. based on the FUMI theory, were used for the optimization. The ϕ–θ plots made it easy to decide optimal CLC-ECD conditions. Using a CLC-ECD system composed of a pump (MP-711i, GL Science), a sample injector fitted with 0.2 μL injection loop (model 7520, Reodyne), an Inertsil ODS-3 column (150 mm × 0.2 mm i.d., 3 μm), the detection limits of 9.76 fg baicalin and 7.03 fg baicalein were obtained at a flow rate of 1.8 μL/min and at the applied potential of +650 mV *vs*. Ag/AgCl.

The chromatographic peak areas of baicalin and baicalein were found to be linearly related to the amounts, ranging from 32.7 fg to 2.23 pg ($r > 0.998$) and 23.4 fg to 1.35 pg ($r > 0.998$), respectively. Determination of baicalin and baicalein in Japanese Pharmacopoeia Scutellaria Root and distribution analysis of baicalin and baicalein in *S. baicalensis* Georgi were carried out by the present CLC-ECD with high sensitivity.

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