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論文内容の要旨

Introduction

Herbal medicines generally exert their therapeutic effects through the synergic action of the multiple bioactive components, which are affected by the varieties of herbs and the medicinal formulas. *Flos Chrysanthemi*, the dried capitulum of *Chrysanthemum morifolium* Ramat. (Compositae), is a well-known crude drug for their therapeutic effects of scattering cold, cleaning heat and toxin properties, and brightening eyes with several cultivated varieties available, in which, four varieties of *C. morifolium* cv. 'Hangju' (HJ), 'Boju' (BJ), 'Chuju' (CJ), and 'Gongju' (GJ) have been recorded into Chinese Pharmacopoeia (2010) as the standard varieties. In contrast, Shuang-Huang-Lian (SHL) preparation, a representative composite formula of traditional Chinese medicine, is comprised of three herbs: *Flos Lonicerae*, *Radix Scutellariae*, and *Fructus Forsythiae*, which is commonly used to treat upper respiratory illnesses caused by viruses or bacterial infections such as tonsillitis, pharyngitis, pneumonia, *etc*. Considering these crude drugs and composite formulas, it is required to simultaneously determine the bioactive components in order to perform the quantitative evaluation of herbal medicines.

A liquid chromatography (LC) with electrochemical detection (ECD) is a valuable analytical method due to its high sensitivity and selectivity for the redox compounds such as caffeoylquinic acids and flavonoids, which are the main bioactive components in *Flos Chrysanthemi* and SHL preparations. As the gradient elution ECD would reduce the sensitivity of the analytes and the single channel isocratic elution ECD is hard to obtain the good separation of the different polarity analytes, the development of multi-channel ECD system was necessary for the quantitative evaluation of herbal medicines. In this study, a novel LC with three-channel isocratic elution ECD (LC-3ECD) system with high sensitivity and efficiency has been developed to simultaneously determine the various redox compounds

by the new design of channel connections and the technique of alternate rotations of switching valves. Moreover, by the analyses of *Flos Chrysanthemi* and SHL preparations as examples, it was shown that LC-3ECD was useful for the quantitative evaluation of herbal medicines.

Chapter 1 The novel LC-3ECD method for the determination of various redox compounds

An LC-3ECD method has been developed to simultaneously determine 7 caffeoylquinic acids of chlorogenic acid (CHA), neochlorogenic acid (NCHA), cryptochlorogenic acid (CCHA), isochlorogenic acid A (ICHA A), isochlorogenic acid B (ICHA B), isochlorogenic acid C (ICHA C), and caffeic acid (CFA) and 2 flavonoids of luteolin 7-O-glucoside (LTG) and luteolin (LT) from *Flos Chrysanthemi*, and 6 caffeoylquinic acids of CHA, NCHA, CCHA, ICHA B, ICHA C and CFA, 4 flavonoids of scutellarin (STL), baicalin (BC), wogonoside (WGD), and baicalein (BCE), and 1 phenylethanoid glycoside of forsythoside A (FTA) from SHL preparations. As shown in Fig. 1, a novel LC-3ECD system has been designed, assembled, and established for the simultaneous determination of various bioactive components in herbal medicines. Through alternately rotating the SV_1 and SV_2 at the different time to change the elution flow way on pre-column, the high polarity compounds, middle polarity compounds, and low polarity compounds retained in pre-column after sample injection, were alternatively eluted into column $_1$, $_2$, and $_3$ by MP_1, MP_2, and MP_3, respectively. Then, the high polarity compounds were fully separated on column $_1$ and determined in detector $_1$. The middle polarity compounds were fully separated on column $_2$ and determined in detector $_2$. The low polarity compounds were fully separated on column $_3$ and determined in detector $_3$. Meanwhile, MP $_1$, $_2$, and $_3$ always flowed through detector $_1$, $_2$, and $_3$, respectively, to keep the isocratic elution in each detection channel in the whole analytical process.

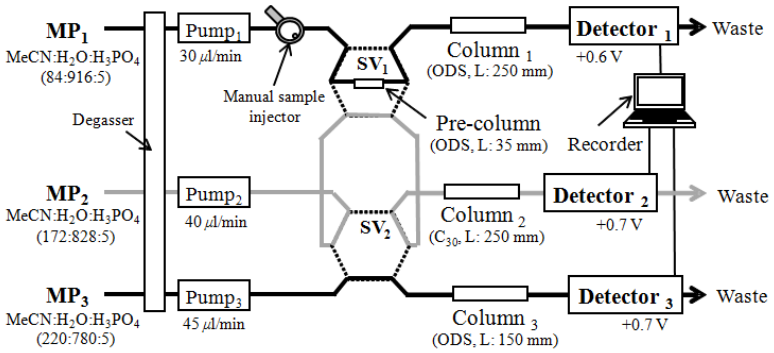

Fig. 1 Block diagram of the present LC-3ECD for the analysis of *Flos Chrysanthemi*. MP $_{1\text{-}3}$, mobile phase; SV $_{1\text{-}2}$, switching valves; MeCN, acetonitrile; H_2O, water; H_3PO_4, phosphoric acid. Composition (v/v/v) of MP $_{1\text{-}3}$, flow rate of MP $_{1\text{-}3}$ (μl/min), length (L, mm) of pre-column and column $_{1\text{-}3}$, and applied potential (V vs. Ag/AgCl) in detector $_{1\text{-}3}$ were described in the figure.

By the present LC-3ECD, the standard mixtures containing 9 compounds of NCHA, CHA, CCHA, CFA, LTG, ICHA B, ICHA A, ICHA C, and LT concerned with *Flos Chrysanthemi* were analyzed under the present system conditions shown in Fig. 1 and the chromatographic separation results were shown in Fig. 2. Furthermore, the standard mixtures containing 11 compounds of NCHA, CHA, CCHA, CFA, FTA, STL, ICHA B, ICHA C, BC, WGD, and

BCE concerned with SHL preparations were also analyzed by the present LC-3ECD to verify the effectiveness and feasibility of the present LC-3ECD. The desirable separation, wide linear range, and high sensitivity, as well as the good precision, were obtained by LC-3ECD. The present LC-3ECD method for determining 9 compounds from *Flos Chrysanthemi* and 11 compounds from SHL preparations has been validated by the standard substances.

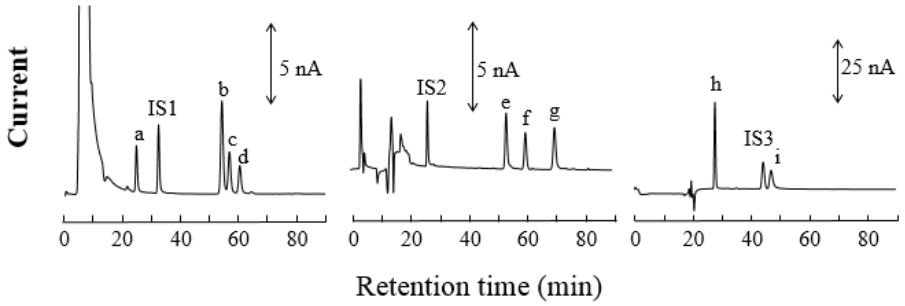

Fig. 2 Chromatograms of mixed standards concerned with *Flos Chrysanthemi*. Peaks: NCHA (a), CHA (b), CCHA (c), CFA (d), LTG (e), ICHA B (f), ICHA A (g), ICHA C (h), LT (i). The left, middle, and right chromatograms were obtained from detector $_1$, $_2$, $_3$, using protocatechuic aldehyde (PAD, IS1), ethyl gallate (EG, IS2), and butyl gallate (BG, IS3) as internal standards, respectively.

Chapter 2 System repeatability estimation in quantitative HPLC-UV and LC-3ECD for herbal medicines by ISO 11843-7

A new methodology to estimate the system repeatability in quantitative HPLC-UV and LC-3ECD for herbal medicines was proposed based on International Organization for Standardization 11843 part 7 (ISO 11843-7), which provides detection limits stochastically. By ISO 11843-7, a real power spectrum was obtained from the baseline noise of a chromatogram by Fourier transform. By the least squared fitting of the model power spectrum to the real power spectrum, the SD of white noise ($\tilde{w}$), and the SD of the Markov process ($\tilde{m}$), and the retention parameter (ρ) of the Markov process were determined as three noise parameters. A measurement RSD of the chromatographic peak, the expression of precision/ system repeatability, which has a peak area (A) or peak height (H), width (*i.e.*, integration domain, k_f), and error of injection volume (I), was obtained by the following equation:

$$\mathrm{RSD}^2 = \frac{k_f \tilde{w}^2}{A^2} + \frac{\tilde{m}^2}{(1-\rho)^2 A^2}\left(k_f - 2\rho\frac{1-\rho^{k_f}}{1-\rho} + \rho^2\frac{1-\rho^{2k_f}}{1-\rho^2}\right) + I^2$$

While using H for determination, A was substituted by H. In an HPLC-UV for determining BC from *Scutellaria Radix*, the stochastically observed RSD of A at 50 μg/ml of BC by ISO 11843-7 was 0.12% (n=1), which was within the 95% confidence interval of the statistically observed RSD by repetitive measurements (n=6), ranging from 0.094% to 0.37%. In an LC-3ECD for determining 9 compounds from *Flos Chrysanthemi*, the stochastically observed RSDs of H at 30 ng/ml of CHA, CCHA, ICHA B, ICHA A, LTG, and LT, 20 ng/ml of NCHA,

10 ng/ml of CFA and ICHA C by ISO 11843-7 (n=1) were also within the 95% confidence intervals of the statistically observed RSDs (n=6) at each concentration of the compounds, respectively. Therefore, ISO 11843-7 was applicable to estimate the system repeatability (precision) in HPLC-UV and LC-3ECD for herbal medicines, contributing to reducing the experimental time for providing a reliable validation.

Chapter 3 Quantitative evaluation of *Flos Chrysanthemi* and SHL preparations by LC-3ECD

After the development of the LC-3ECD system by the new design of channel connections, the verification of its effectiveness by the standard substances, and the reconfirmation of its precision by ISO 11843-7, its specificity, repeatability, and accuracy were validated by using the real samples. Then, 9 bioactive components of NCHA, CHA, CCHA, CFA, LTG, ICHA B, ICHA A, ICHA C, and LT from *Flos Chrysanthemi* and their sulfur-fumigated products were simultaneously determined by LC-3ECD. Significant differences were found among four cultivated varieties of 'HJ', 'BJ', 'CJ', and 'GJ' samples and the sulfur-fumigated varieties of 'S-HJ' and 'S-BJ' samples by the content determination and principle component analysis (PCA). Based on the content determination, nearly 60% of LTG and more than 47% of caffeoylquinic acids were lost during the sulfur fumigation process. Sulfur fumigation showed a destructive effect on *Flos Chrysanthemi*. Concerning the PCA results of 'HJ', 'BJ', 'CJ', and 'GJ' samples shown in Fig. 3, PC_1 and PC_2 explained 68.8% and 19.1% of the variation, respectively. 87.9% of the total variation was illustrated by two PCs. The samples of 'HJ', 'BJ', 'CJ', and 'GJ' were clearly classified into four groups on the scores plot (Fig. 3A). The weights of 9 analytes combined to form the PCA scores were shown on the loadings plot (Fig. 3B). The similarity and inhomogeneity of 9 analytes in four cultivated varieties of *Flos Chrysanthemi* were clearly exhibited in Fig. 3A and Fig. 3B.

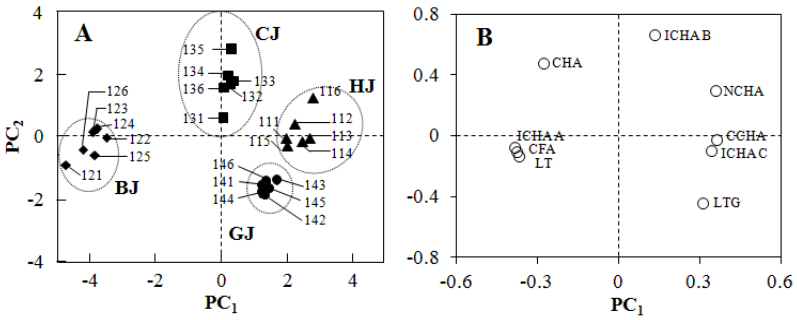

Fig. 3 PCA results obtained from the content data set of 'HJ', 'BJ', 'CJ' and 'GJ' samples. (A) the scores plot; (B) the loadings plot.

As next real samples, 11 bioactive components of NCHA, CHA, CCHA, CFA, FTA, STL, ICHA B, ICHA C, BC, WGD, and BCE from 14 batches of SHL oral liquid and 12 batches of SHL lyophilized powder for injection were simultaneously determined by the present LC-3ECD. The contents of these 11 components differed greatly among the different batches of SHL samples. Therefore, the multiple components determination by the present LC-3ECD could reflect the quality of herbal medicines, comprehensively.

Conclusions

In this study, a novel LC-3ECD method has been developed for the simultaneous determination of various bioactive components from *Flos Chrysanthemi* and SHL preparations with high sensitivity and efficiency. It is reliable, applicable and superior for the quantitative evaluation of herbal medicines. In addition, ISO 11843-7 was successfully applied to estimate the system repeatability of quantitative HPLC-UV and LC-3ECD. It is a very helpful methodology for the system repeatability estimation of HPLC during the quantitative evaluation of herbal medicines.

Publications

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(2) Chen L., Kotani A., Kusu F., Wang Z., Zhu J., Hakamata H., *Chem. Pharm. Bull.*, **63(1)**, 25-32 (2015).

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論文審査の結果の要旨

本論文は、電気化学検出 HPLC（HPLC-ECD）の特長である高い感度と特異性に着目し、種々の電極反応を検出原理として活用することによって、薬物と生体成分の新たな高感度汎用分析法の開発を行ったものである。酸化還元物質の定量に際しては、電解条件を制御することによって、電極表面で生じる測定対象物質の酸化電流又は還元電流を計測し、感度と特異性の向上を図っている。酸、或いは塩基の定量に際しては、電子授受反応のメディエータとして用いたキノンの還元前置波、或いはトロロックスの酸化前置波に由来する電流を測定し、酸及び塩基の特異的な測定システムを構築している。以上のように、酸化還元物質、酸、塩基、それぞれを特異的に検出するための電極反応を検出モードとして測定システムに組み入れ、高感度化、高精度化、適切な試料前処理法の設定を行い、実試料中の薬物定量における HPLC-ECD の有用性を明らかにしている。

第 1 章では、腎毒性を持つアリストロキア酸（AA）の生薬への混入監視法を開発している。測定対象の AA が電解還元することを見出し、その還元反応を検出に利用する AA の HPLC-ECD を構築した。HPLC 条件として、分離には ODS カラム、移動相にはメタノール/水/リン酸（65:35:0.5, v/v/v）混液を選定した。本法は、日局のサイシンの純度試験（アリストロキア酸 I（AA1））で定められている吸光検出 HPLC（HPLC-UV）と比較して、夾雑ピークの影響を受けにくく、特異性の点で優れていた。さらに、本法の AA1 の検出限界は 3.4 ng/mL であり、生薬中の AA1 含量が 85 ng/g 以下であることを確認可能であった。これらの結果から、日局の方法より厳重な AA の監視法として本法が適用できることを示した。

第 2 章では、バルプロ酸（VPA）、5 種の遊離脂肪酸（FFA）、血糖（BG）の複合動態解析法を開発している。VPA 及び FFA の検出にキノンの還元前置波に由来する電流計測が利用できることを示し、本検出モードを組み込んだ 2 流路系の HPLC-ECD をそれぞれ構築した。VPA の分離には C30 カラム、移動相にはアセトニトリル/水（70:30, v/v）混液、FFA の分離には C30 カラム、移動相にはアセトニトリル/エタノール（90:10, v/v）混液を選定した。VPA の HPLC-ECD、FFA の HPLC-ECD、及び血糖測定器を用いた in vivo 複合動態解析では、健常ラット、マルトース負荷ラット、アロキサン糖尿病ラットにおいて、尾静脈より経時的に採血した 50 □L の血液で VPA、FFA、BG の詳細な時間-濃度プロファイルを取得でき、VPA の薬物動態（PK）パラメータの算出が可能であった。得られた結果から、アロキサン糖尿病ラットでは VPA の代謝と排泄が促進されていることを見出した。以上から、VPA の薬物動態分析と、in vivo における糖脂質代謝調節への VPA 投与の影響を同時に把握するための分析法として、本法が有用であることを示した。

第 3 章では、テオフィリンの薬物動態分析法を開発している。塩基としてテオフィリンを扱い、その検出にトロロックスの酸化前置波に由来する電流計測が利用できる

ことを示し、2 流路系の HPLC-ECD を構築した。HPLC 条件として、分離にはシリカゲルカラム、移動相にはアセトニトリルを選定し、テオフィリンを高感度かつ特異的に検出できる方法として確立した。本法によって得たラット血中テオフィリンの時間-濃度プロファイルを基に、テオフィリンの PK パラメータが算出され、本法がテオフィリンの薬物動態分析に適用可能であることを明らかにした。

以上、本研究は HPLC-ECD の特長を踏まえ、測定対象物質毎に適切な電極反応を利用する検出モードを組み込んだ HPLC システムを構築し、高感度且つ特異的な定量分析法を開発している。生薬への AA の混入監視法として、日局の HPLC-UV 法よりも感度と精度に優る方法が開発されており、社会的な貢献が期待できる。また、VPA の薬物動態と、グルコース及び脂肪酸代謝への VPA 投与の影響を in vivo で観察するための複合動態解析法が提案されており、アロキサン糖尿病ラットでは VPA の代謝と排泄が促進されるとの新知見を得ている。更に、微量の血液試料でテオフィリンの薬物動態分析を行うことができる高感度分離定量法が開発されている。以上のように本論文は、薬学領域の研究の進展並びに実践に十分寄与できる新規分析法の開発を行ったものである。よって、本論文は、博士（薬学）の学位論文として十分な価値を有するものであると判断する。