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Prediction of internal standards in reversed-phase liquid chromatography
II. Selectivity optimization and internal standard prediction for the quantitation of estradiol and levonorgestrel in a transdermal drug delivery formulation based on the linear solvation energy relationships

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Abstract

This paper describes the results of selectivity optimization and internal standard prediction for the quantitation of estradiol and levonorgestrel in transdermal patches by reversed-phase liquid chromatography (RPLC) based on the linear solvation energy relationships (LSERs). The patch samples are prepared by swelling with acetonitrile (ACN) and the separation is performed by Zorbax Eclipse XDB ODS columns. A proper retention range is first determined with a binary mobile phase of ACN and water based on the general resolution equation. The interference to estradiol from a levonorgestrel impurity is then eliminated by a ternary mobile phase of acetonitrile–methanol–water with a composition predicted by LSERs. When the resolution is optimized and the ''open window'' in the chromatogram for an internal standard is selected, LSERs are used to predict the candidate compounds to be evaluated as the internal standard. The approach described in this study can be used, in general, to considerably improve the efficiency of RPLC method development, particularly for neutral samples. Finally, the LSER approach for the selectivity optimization is compared with a statistical response surface methodology (RSM) based on a central composite design (CCD) in terms of the effectiveness and number of experiments. It is concluded that, although the predicted mobile phase composition to achieve the desired selectivity is about the same, the LSER approach is more efficient and fewer experiments are required.

Keywords: Internal standards; Linear solvation energy relationships; Transdermal drug delivery; Response surface; Selectivity optimization; Estradiol; Levonorgestrel

1. Introduction

Transdermal delivery of estrogen has been the preferred system for hormone replacement therapy (HRT) [1]. Estradiol is usually used in the system to treat postmenopausal symptoms such as hot flashes

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and to prevent osteoporosis [2]. It is also reported to have cardioprotective effects, to relive urogenital atrophy, and to decrease urinary incontinence. Levonorgestrel (a progesterone) can be included in the formulation for opposing negative effects of estrogen to avoid endometrial hyperplasia and possible malignant transformation [3]. The structures of estradiol and levonorgestrel are shown in Fig. 1. A patch system consisting of both estradiol and levonorgestrel has been evaluated at 3M.

The quantitation of both estradiol and levonorgestrel in the patches is performed by RPLC. To ensure the accuracy of the separation, two major issues are emphasized during the method development. They are the stability-indicating nature of the method and robustness of the chromatography. Stability-indication refers to the absence of interference to the major component from drug impurities/degradation products or excipient and its impurities. This is related to the resolution optimization during method development. The robustness of a separation is also very critical because the method may be used in different laboratories by different people at different locations.

The development of a chromatographic method is usually and best guided by the general resolution equation (see Eq. (1)) [4]:

$$R_s = \left(\frac{\sqrt{N}}{4}\right)_3 (\alpha - 1)_2 \left(\frac{k'}{1+k'}\right)_1 \quad (1)$$

where R_s is the resolution; N is the column efficiency; α is the selectivity, and k' is retention factor. The subscript number after each parenthesis suggests the order for resolution optimization. Proper retention should be optimized first for resolution requirement, followed by selectivity adjustment. The optimization of chromatographic selectivity is the most challenging part of method development due to any potential interference to major drug component. Although a trial-and-error method is often used, quite a few techniques can be applied for a systematic optimization of selectivity. These techniques can be classified into two categories in general, namely the chemometric and model methods. The chemometric methods include the overlapping resolution maps, factorial design, and response surface methodology. The model methods include the computer-based optimization (e.g., DryLab) and optimization based on structural parameters (e.g., the LSER method). A review article on optimization of selectivity in chromatography and capillary electrophoresis was published recently [5].

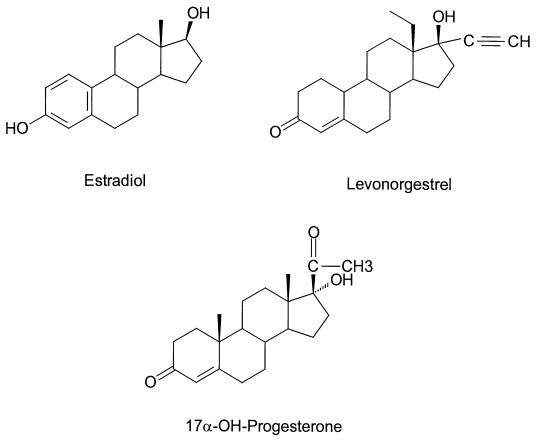

Fig. 1. The structures of estradiol, levonorgestrel, and 17α-OH-progesterone.

The robustness of chromatographic methods can be improved in several ways. They include, for example, the use of better instrumentation, improvement in the skills of analytical chemists, and incorporation of procedures to compensate small variations in instrumental and sample preparation procedures. The internal standard technique is one of the approaches for the improvement of method robustness. The rationale for use of an internal standard has been thoroughly examined recently [6]. However, the selection of an internal standard compound is usually through an empirical approach. A systematic method for the rapid and accurate prediction of internal standards has just been published [6].

This paper describes the use of LSERs to improve the efficiency of selectivity optimization and the selection of the internal standard during the method development for estradiol and levonorgestrel formulation. LSER is a type of quantitative structure–activity relationship, and relates the chromatographic retention to the physicochemical properties (also called descriptors) of solutes. LSERs have been mainly used for stationary phase characterization, its use in selectivity optimization is rather limited [5]. The difficult part of this methodology is the determination of properties of solutes for chromatographic method development for average chroma-

tographers. However, with the recent advance in the calculation of the descriptors of compounds by commercial software, the approach can be easily used for method development now.

Although there are many studies on the separation of steroids in the literature, the focus of the study is the use of LSERs for the fast optimization of selectivity and the prediction of internal standard candidates. A few most relevant references are cited here from a quick search of literature dated back to 1993 [7–14]. In particular, the structure–retention relationships have been used to predict retention based on structural parameters [13,14]. The USP monograph also includes the quantitation of estradiol and levonorgestrel in tablets [15]. Finally, the LSER method is compared with a statistical RSM to determine the relative effectiveness and the experimental requirement for the optimization of selectivity.

2. Theoretical consideration

2.1. Selectivity formulated by linear solvation energy relationships

The optimization of selectivity is guided by the use of LSERs ([16–17] and Refs. therein). Based on LSERs, the retention in liquid chromatography is expressed as:

$$\mathrm{Log}(k') = \mathrm{Log}\, k'_0 + m\frac{V}{100} + s\pi + a\alpha + b\beta \qquad (2)$$

where $\mathrm{Log}(k'_0)$ is the regression constant, V is the solute McGowan characteristic molar volume; π is the solute dipolarity/polarizability; α and β are the solute ''overall'' or ''effective'' hydrogen bond acidity and basicity, respectively; and m, s, a and b are the coefficients determined by the multivariable linear regression analysis. Parameters V, π, α and β are called solutes' descriptors, and they represent the physicochemical properties of solutes. Solutes' descriptors are available for more than 4000 compounds and further values can be obtained by parameter estimates [18–24] or computed by commercial software (Sirius Analytical Instruments, East Sussex, UK).

The selectivity ($\alpha_{2/1}$) of two solutes can be derived from Eq. (2) as:

$$\begin{aligned}\mathrm{Log}(\alpha_{2/1}) &= \mathrm{Log}\left(\frac{k'_2}{k'_1}\right) \\ &= \frac{(V_2 - V_1)}{100} + s(\pi_2 - \pi_1) + a(\alpha_2 - \alpha_1) \\ &\quad + b(\beta_2 - \beta_1) \qquad (3)\end{aligned}$$

where subscripts 1 and 2 denote solute 1 and 2, respectively; and k'_2 and k'_1 are their retention factors. Additionally, the selectivity change of two solutes by two different mobile phase compositions can be derived from Eq. (3) as follows:

$$\begin{aligned}\mathrm{Log}\left(\frac{\alpha_{2/1\text{-B}}}{\alpha_{2/1\text{-A}}}\right) &= (m_\mathrm{B} - m_\mathrm{A})\frac{(V_2 - V_1)}{100} \\ &\quad + (s_\mathrm{B} - s_\mathrm{A})(\pi_2 - \pi_1) \\ &\quad + (a_\mathrm{B} - a_\mathrm{A})(\alpha_2 - \alpha_1) \\ &\quad + (b_\mathrm{B} - b_\mathrm{A})(\beta_2 - \beta_1) \qquad (4)\end{aligned}$$

where $\alpha_{2/1\text{-B}}$ and $\alpha_{2/1\text{-A}}$ are the selectivity by two different mobile phase compositions (denoted as A and B); m_B and m_A, s_B and s_A, a_B and a_A, and b_B and b_A are the LSER coefficients obtained for the two mobile phase compositions.

Eq. (3) will be used to predict the internal standard candidates, as will be explained below. However, Eq. (4) can be used to predict the dependence of chromatographic selectivity on the mobile phase composition.

2.2. Prediction of selectivity change with mobile phase composition

The prediction of selectivity change consists of several steps, and they are summarized in Table 1. Each step is described in detail below.

2.2.1. Step 1: optimize initial mobile phase composition

As indicated in the introduction, the development of a chromatographic separation is best approached based on the general resolution equation (Eq. (1)). The separation of estradiol and levonorgestrel is initially optimized to a reasonable retention range by ACN–water binary mobile phase (40% ACN by volume). However, at this mobile phase composition,

Table 1
Steps used to predict chromatographic selectivity change

Step	Action
1	Determine the mobile phase composition for proper retention range. The initial mobile phase is binary ACN–water
2	Determine isoelutropic compositions of the ternary mobile phase of ACN–MeOH–water. Use Eq. (5) to compute the volume fraction of MeOH for different ACN composition
3	Determine LSER coefficients based on the literature data for ODS column
4	Determine solutes' descriptors for the selectivity calculations
5	Calculate the change in selectivity with mobile phase composition by Eq. (4)

there is interference to estradiol from a levonorgestrel impurity, ketolevonorgestrel. Therefore, the resolution between the two compounds will be improved by adjusting the selectivity (second step in Eq. (1)).

There may be several ways to change the selectivity. However, the selectivity is improved in this study by ternary mobile phases of similar strength (isoelutropic), and methanol (MeOH) is the third solvent.

2.2.2. Step 2: calculation of the mobile phase compositions of isoelutropic strength

The binary ACN–water is regarded as the initial mobile phase. MeOH is used as the third solvent to improve the selectivity. Based on the solubility parameter theory, MeOH composition (ϕ_{MeOH}) is related to the composition of ACN (ϕ_{ACN}) in the ternary mobile phase (to keep approximately constant retention) as follows [25]:

$$\phi_{MeOH} = \frac{(\phi^*_{ACN} - \phi_{ACN})(\delta_{ACN} - \delta_{water})}{(\delta_{MeOH} - \delta_{water})} \quad (5)$$

where ϕ^*_{ACN} denotes ACN composition in the initial mobile phase; and δ denotes the solubility parameters for each solvent. The solubility parameters for ACN, MeOH, and water are 13.14, 15.85, 25.52 $((cal/cm^3)^{1/2})$, respectively [25].

2.2.3. Step 3: calculation of LSER coefficients

The LSER coefficients for ODS columns can be computed directly based on the data from literature. Each coefficient is related to the mobile phase composition as follows [26]:

$$y = A\phi_{MeOH} + B\phi_{ACN} + C\phi_{water} + D\phi_{MeOH}\phi_{ACN} + E\phi_{MeOH}\phi_{water} + F\phi_{ACN}\phi_{water} \quad (6)$$

where y is a LSER coefficient; and A–F are the constants determined in the literature. All mobile phase compositions are in volume fraction in Eq. (6). LSER coefficients at different compositions of isoelutropic strength are then computed based on the compositions and constants. Constants for MeOH–tetrahydrofuran (THF)–water ternary system are also available [26].

It is noted that the regression constant ($\mathrm{Log}(k'_0)$) in Eq. (2) cannot be computed and it is related to the carbon loading of the column. However, we are making predictions based on the relative retention (selectivity), the effect of the constant is eliminated (see Eqs. (3) and (4)).

2.2.4. Step 4: estimation of solute descriptors

There are at least three ways to obtain solutes' descriptors. The first is to search the literature data [18–24]. Solute descriptors are already available for more than 4000 compounds. The second approach is to compute them by commercial software (Sirius Analytical Instruments). The third method is to estimate by analogy or fragment addition [21,27–30].

The descriptors of estradiol are available from literature [27]. However, the descriptors of ketolevonorgestrel are estimated based on those of 17α-hydroxyprosterone [27] because they have the same number of major functional groups and share very similar structures. The structure of ketolevonorgestrel is essentially the same as levonorgestrel with an additional keto functional group on the molecule. The structure of 17α-hy-

Table 2
Estimation of the descriptors of the solutes

Compounds	Solute descriptors			
	π	α	β	$V/100$
Estradiol[a]	3.30	0.88	0.95	2.199
17α-OH-Progesterone[a]	3.35(2.35)	0.25(0.34)	1.31(1.19)	2.680
Ketolevonorgestrel[b]	3.35(2.35)	0.25(0.34)	1.31(1.23)	2.594[c]
Difference (ketolevonorgestrel–estradiol)	0.05	−0.63	0.36	0.395

[a] The descriptors of estradiol and 17α-OH-progesterone are taken from Ref. [27]. The solute volumes ($V/100$) are computed by the method of McGowan [35]. The values in the parentheses indicate the predicted by Absolv Software (Sirius Analytical Instruments).
[b] The descriptors (except for the volume) for ketolevonorgestrel are assigned the same as those of 17α-OH-progesterone. The values in the parentheses indicate the predicted by Absolv Software (Sirius Analytical Instruments).
[c] The volume descriptor is computed separately by the McGowan method [35].

droxyprosterone is also shown in Fig. 1, and the estimation in the descriptors of ketolevonorgestrel is shown in Table 2.

2.2.5. Step 5: calculation of selectivity change with mobile phase composition

After we obtained the differences in descriptors and LSER coefficients at different mobile phase compositions, the change in selectivity with the composition is predicted by Eq. (4).

2.3. Prediction of internal standard candidates

The prediction of internal standard candidates is published recently [5]. It is based on either absolute or relative retention. For convenience, the relative retention method is briefly described here because it is used in this study.

When the mobile phase composition and type of column are finalized during the method development, a decision will be made on the position (retention) of the internal standard based on the ''open window'' of the chromatogram. Prior to the prediction of the internal standard, the retention time of one or two reference solutes such as 4-nitrotoluene (used in this study) and phenol are collected. The selectivity of the internal standard position relative to the reference solute(s) is used to predict the internal standard candidates (Eq. (3)). The purpose of the reference solute is to compensate the difference among the different ODS columns, for example. The selectivity is the criterion to meet for the internal standard compound. Based on the LSER coefficients obtained for the final mobile phase composition, we compute the selectivity of many compounds in a database relative to 4-nitrotoluene based on their descriptors (V^i, π^i, α^i and β) and the reference solute's descriptors (V^R, π^R, α^R and β^R). The selectivity is then sorted in either increasing or decreasing order. Compounds that meet the desired selectivity within a narrow range are considered the candidates for the internal standard. Finally, the selected compounds are tested experimentally under the same chromatographic conditions. The solutes that best match the desired selectivity are selected for the internal standard. The database can be established by scanning the tabulations in literature [31] into a spreadsheet program (available from the author) or obtained commercially (Sirius Analytical Instruments).

Finally, it should be emphasized that other properties of compounds such as the UV absorbency profile, purity, toxicity should also be taken into account in the selection of the internal standard.

3. Experimental

3.1. Chromatographic instrumentation and separation conditions

All experiments were performed on HP 1090 liquid chromatograph equipped with a ternary pump, autosampler, and diode array detector (Agilent Technologies, Wilmington, DE, USA). A computer-based

workstation (Chemstation, Agilent) was used not only to control the instrumentation, but also to collect chromatographic data.

Zorbax Eclipse columns (150×4.6 mm, 5 μm) (Agilent Technologies) are used for the separation. The mobile phase is initially optimized to 40% ACN–60% water to provide sufficient retention. Then ternary mobile phase of ACN–MeOH–water is used to improve the selectivity. Two ternary mobile phases are experimentally evaluated, and they are composed of 35:7:58 and 30:15:55% of ACN–MeOH–water, respectively. The flow-rate is kept at 2 ml/min, and the injection volume is always 10 μl. The detection wavelength is at 225 nm for the proper detection of both estradiol and levonorgestrel. The system hold-up time is 0.75 min by ACN disturbance peak. It is noted that all mobile phase compositions are in volume percent in this study, unless otherwise indicated.

3.2. Samples and internal standard candidates

Three major patch samples are prepared. The estradiol and levonorgestrel sample is prepared by swelling 30-cm^2 transdermal patches (1.5% estradiol and 1.25% levonorgestrel) by ACN solvent. Two other patch samples are prepared by swelling with solutions of ketolevonorgestrel and 4-*sec*.-butylphenol (the solvent is ACN). The volume of solvent and solutions is 25 ml per patch. Both solutions are prepared in ACN. The concentration of ketolevonorgestrel is about 0.15 mg/ml, and that of 4-*sec*.-butylphenol about 1 mg/ml. The patches are shaken for 4 h for a full dissolution. The supernatants are then collected for injection.

Table 3 lists the candidate compounds used for the selection of the internal standard for the separation. They are all dissolved in ACN and their concentrations ranged 1–2 mg/ml. All chemicals are obtained from Aldrich (Milwaukee, WI, USA).

3.3. Statistical design and experiments for the response surface methodology

3.3.1. Choice of experimental design

As mentioned early, other systematic approaches for selectivity optimization are also available [5,32–34]. To compare the effectiveness and experimental

Table 3
Internal standard candidates used in the study

Candidate[a]	Predicted selectivity[b] (α)	Measured selectivity[c] (α)	Measured retention time[d] (min)	Remarks
Estradiol			5.76(0.35)	
Levonorgestrel			12.46(0.02)	
4-Nitrotoluene	1		6.10	Reference solute
Position of I.S.	1.73[e]			Desired selectivity
4-*sec*.-Butylphenol[f]	1.76	1.78	10.30(0.04)	Best match in selectivity
4-*tert*.-Butylphenol[f]	1.76	1.45	8.50	
2-*tert*.-Butylphenol	1.56	1.44	8.46	
Benzyl bromide	1.52	1.56	9.10	
2-*sec*.-Butylphenol	1.50	2.04	11.68	
Bromobenzene	1.51	1.79	10.32	
p-Chloroanisole	1.41	1.59	9.26	

This table summarizes the compounds used in searching for the internal standard.
[a] Internal standard candidates.
[b] Predicted selectivity relative to 4-nitrotoluene.
[c] Measured selectivity.
[d] Retention times obtained by the column and flow-rate of 2 ml/min. The values in the parentheses indicate RSD(%) of retention times.
[e] Prediction criterion.
[f] 4-Butylphenol is the predicted compound. Two isomers are selected for testing.

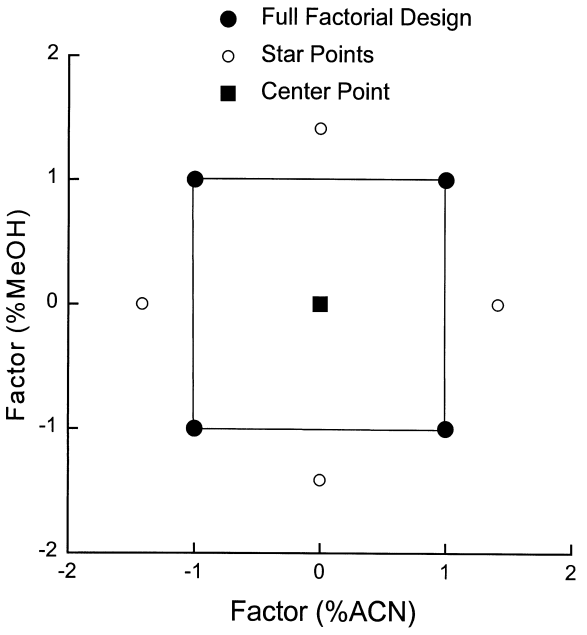

Fig. 2. Illustration of the central composite design used for the response surface methodology.

procedure of the LSER approach for the selectivity optimization with other methods, a statistical RSM for the selection of the mobile phase composition is performed. Our goal is to determine the change in selectivity between ketolevonorgestrel and estradiol with mobile phase compositions. A CCD with two factors (%ACN and %MeOH) and five levels is selected. It is build up of a full factorial 2^k (k = the number of factors) design to which a star design is added [32,34]. The length of the arms of the star is $\alpha = \sqrt{2}$. The CCD is completed by addition of a center point. The principal representation of the CCD is shown in Fig. 2. The total number (N) of experiments with k factors is given by:

$$N = 2^k + 2k + c \quad (7)$$

where the first term is related to the full factorial design, the second to the star points and the third to the center point. For two factors ($k = 2$) to be considered, at least $4+4+1=9$ experiments are necessary. If five replicates of the center point is used. The total of number of experiments is 13. The replicates of the center of the domain can be used to estimate the experimental variance.

Because we already know that a small amount of MeOH can improve the selectivity (see Section 4), the range of ACN concentration is selected from 30 to 50% and that of MeOH from 5 to 20%. Table 4 shows the design by Minitab (State College, PA,

Table 4
The central composite design table

Standard order[a]	Run order[b]	ACN (%, v/v)	MeOH (%, v/v)	t_{Keto} (min)	t_{E2} (min)	α[c]	t_{Levo} (min)
6	1	54.1	12.5	1.31	1.31	1	2.06
7	2	40	1.9	3.59	3.59	1	7.96
9	3	40	12.5	2.55	2.55	1	4.99
13	4	40	12.5	2.54	2.54	1	4.97
12	5	40	12.5	2.53	2.53	1	4.95
11	6	40	12.5	2.53	2.53	1	4.93
4	7	50	20	1.24	1.28	0.967	1.93
1	8	30	5	8.43	9.36	0.901	22.32
10	9	40	12.5	2.44	2.52	0.969	4.91
5	10	25.9	12.5	8.63	10.63	0.812	24.14
3	11	30	20	3.64	4.29	0.849	8.67
2	12	50	5	1.80	1.74	1.039	3.17
8	13	40	23.1	1.68	1.82	0.922	3.05

The design is performed by Minitab. ACN ranges from 30 to 50% and MeOH from 5 to 20%. Abbreviations: Keto, ketolevonorgestrel; E2, estradiol; and Levo, levonorgestrel.

[a] Standard order.

[b] Experimental order.

[c] Selectivity is defined relative to estradiol.

USA). It is noted in Table 4 that the run order of the experiments is randomized.

3.3.2. Statistical calculations

The responses of the experiments are the retention time (t_{Levo}) of levonorgestrel (as an indication of the analysis time) and the selectivity ($\alpha = k'_{ketolevonorgestrel}/k'_{estradiol}$) between ketolevonorgestrel and estradiol. The responses are fitted by a so-called response surface regression including second-order and interactions terms:

$$t_{Levo}(\text{or } \alpha) = Y + Ax_{ACN} + Bx_{MeOH} + Cx_{ACN}^2 + Dx_{MeOH}^2 + Ex_{ACN}x_{MeOH} \quad (8)$$

where Y, A, B, C, D, and E are coefficients, and x_{ACN} and x_{MeOH} are the volume percents of ACN and MeOH.

After the coefficients are determined, the equation of levonorgestrel retention time will be used to calculate the mobile phase compositions that provide about constant retention time. If ACN concentration is selected, the corresponding MeOH concentration for a constant retention time can be determined by numerically solving the quadratic equation (Eq. (8)):

$$x_{MeOH} = \frac{-(Ex_{ACN} + B) - \sqrt{(Ex_{ACN} + B)^2 - 4D(Y + Ax_{ACN} + Cx_{ACN}^2 - t_{Levo})}}{2D} \quad (9)$$

In this study x_{ACN} varies from 28 to 40%. Then x_{ACN} and x_{MeOH} are used to compute the selectivity (Eq. (8)). All calculations and manipulations are performed by Excel.

3.3.3. Experimental procedure on RSM

The LC experiments are performed based on Table 4. Except for the variation in mobile phase compositions, other conditions remain the same as above. The sample contains all three components. Duplicate injections are made at each condition, and the average retention times are used to compute the selectivity. The selectivity and levonorgestrel retention data are then fitted to Eq. (8). Table 4 also includes the retention and selectivity results.

4. Results and discussion

4.1. Optimization of retention range

To obtain reproducible separation of both estradiol and levonorgestrel, a good retention range is desired. We began the adjustment of retention by a stepwise change of mobile phase composition of ACN–water on Zorbax Eclipse XDB ODS column. This column is selected based on the properties of the solutes. We began the separation with 80% ACN (by volume), and stepwise finalized to 40% ACN. The chromatogram obtained is shown in Fig. 3. The retention times of estradiol and levonorgestrel are 3.9 ($k' = 4.2$) and 8.7 ($k' = 10.6$) min, respectively.

When the retention range is finalized, we evaluated the interference of drug-related impurities to the major components. It has been observed that a levonorgestrel impurity, ketolevonorgestrel (an oxidation product with an additional keto group), is

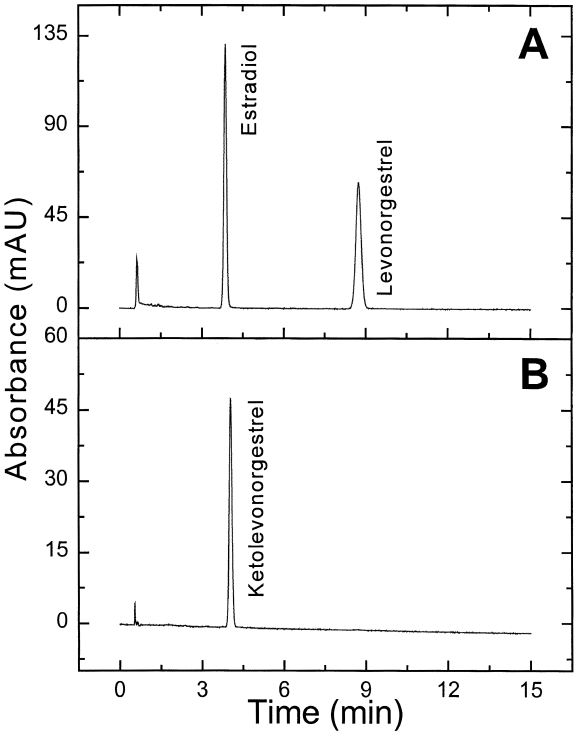

Fig. 3. Illustration of the chromatograms obtained for a patch sample (A) of estradiol and levonorgestrel and ketolevonorgestrel (B) at 40% ACN and 60% water.

co-eluted with estradiol, and its chromatogram is also included in Fig. 3. The retention time of ketolevonorgestrel is 4.0 min ($k' = 4.3$). The interference to a major drug component is a very important issue because it affects the accuracy of the quantitation. Accordingly, an improvement in selectivity between estradiol and ketolevonorgestrel is needed, hopefully, without a major change in retention.

4.2. Optimization of selectivity of estradiol and ketolevonorgestrel

The simplest way to improve the selectivity is to further change the composition of ACN in the mobile phase. Fig. 4 shows the chromatograms obtained at 30 and 50% ACN. It can be seen in Fig. 4A that, even though a baseline resolution is not achieved at 50% ACN, the retention range is undesirable (k' of estradiol is about 1.5). Fig. 4B shows that, although a baseline resolution is nearly obtained at 30% ACN, the analysis time is too long (at least 32 min). Because ketolevonorgestrel switches elution order at this composition, a complete baseline resolution cannot be achieved by adjusting ACN–water mobile phase (between 30 and 50% ACN) without an excessive run time. A different direction is needed.

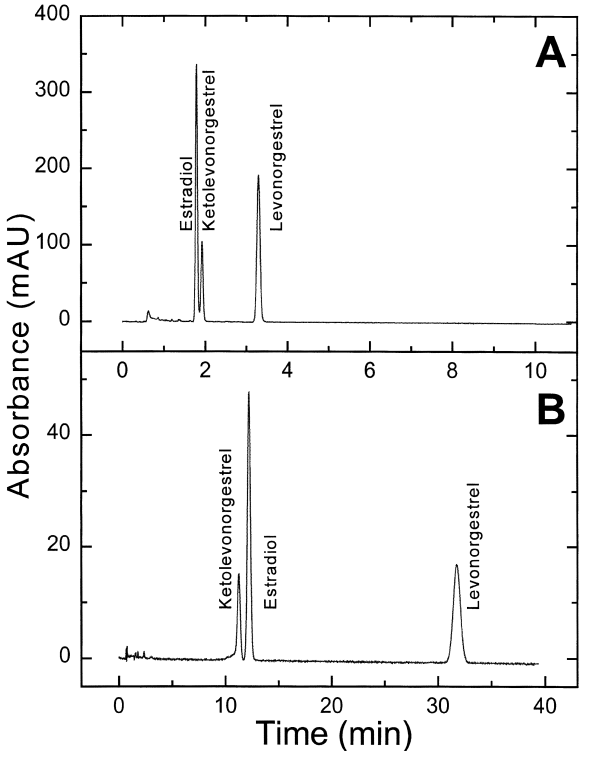

Fig. 4. Illustration of the chromatograms obtained at 50% (A) and 30% (B) ACN. The sample contains estradiol, ketolevonorgestrel and levonorgestrel.

The next simple procedure is to add a third solvent such as MeOH or THF to the mobile phase to improve the selectivity. However, if a systematic method is not used, a proper selection of a ternary mobile phase to provide the desired selectivity will require many trial-and-error experiments. Although many systematic methods are available, we will focus on the use of LSERs for selectivity optimization. A theoretical approach such as LSERs will certainly assist in the selection of the mobile phase composition. The purpose in this case is to use LSERs to evaluate if MeOH will improve the selectivity. Furthermore, if the addition of MeOH can improve their selectivity, can LSERs predict the approximate mobile phase composition?

To predict the change in selectivity, several steps are needed, and they are described in Table 1 and the theoretical section. First, we want to keep the eluting strength of the mobile phase approximately the same (isoelutropic) as the composition changes. Taking 40% of ACN as the initial composition, Eq. (5) is used to calculate MeOH volume fraction, as the composition of ACN decreases. The compositions computed should provide similar strength to 40% ACN. Fig. 5A shows the composition of ACN and MeOH that constitute the isoelutropic line.

Next, the dependence of LSER coefficients on the mobile phase composition is determined. The coefficients at the different mobile phase compositions in Fig. 5A are computed by Eq. (6), and they are used in Eq. (4) to compute the selectivity change. The initial LSER coefficients are indicated in Table 5.

We also need to know the descriptors of estradiol and ketolevonorgestrel to perform the calculations in Eq. (4). Table 2 summarizes the estimation of the descriptors of the two solutes. It is noted in Table 2 that the descriptors for ketolevonorgestrel are not available from the literature, and the descriptors of a similar compound (17α-OH-progesterone) are used to assign π^*, α and β. The volume of the solute is

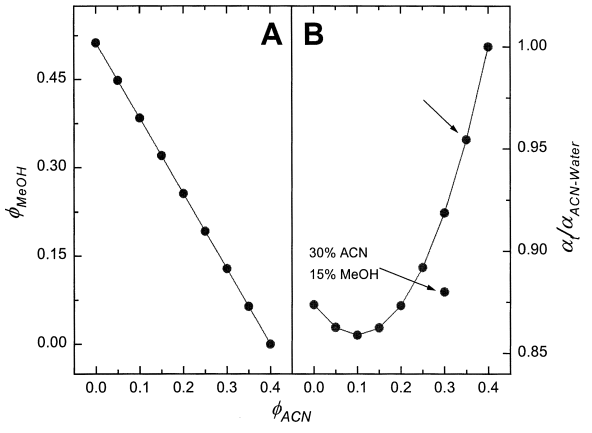

Fig. 5. Illustration of the isoelutropic line (A) and selectivity change (B) with the mobile phase composition. The two arrows in (B) show the selectivity evaluated experimentally. The mobile phase compositions of 30:15:55% of ACN–MeOH–water slightly deviates from the isoelutropic line.

computed by the McGowan method [35]. Also included in Table 2 are the differences in the descriptors.

Substituting the differences in the descriptors (Table 2) and initial LSER coefficients (Table 5) into Eq. (4), the change in selectivity relative to the initial mobile phase (reference mobile phase) can be described as follows:

$$\mathrm{Log}\left(\frac{\alpha_{\mathrm{Ternary}}}{\alpha_{\mathrm{ACN-Water}}}\right) = 0.395(m_t - 1.963) + 0.05(s_t + 0.221) - 0.63(a_t + 0.544) + 0.36(b_t + 1.942) \quad (10)$$

where m_t, s_t, a_t, and b_t are the LSER coefficients at different ternary mobile phase compositions. Subscript t refers to the ternary mobile phase. The selectivity is defined relative to estradiol. It is noted that the selectivity at 40% ACN is unity, Eq. (10) is essentially the absolute change in selectivity with the mobile phase composition. Eq. (10) is the link between the mobile phase composition and change in selectivity through the LSER coefficients. The results of the calculation are shown in Fig. 5B. It can be seen from Fig. 5B that the addition of MeOH is expected to improve the selectivity between estradiol and ketolevonorgestrel. For example, the selectivity changes from 1 to 0.955 when the mobile phase changes to a composition of 35:7:58% of ACN–MeOH–water. The selectivity changes to 0.92 when the mobile phase contains approximately 30:13:57% of ACN–MeOH–water. If the mobile phase contains 30:15:55% of ACN–MeOH–water (slightly deviates from the isoelutropic line), the predicted selectivity is 0.88.

To confirm the calculations experimentally, chromatograms are obtained by the two ternary mobile phase compositions, and they are shown in Fig. 6A (35:7:58% of ACN–MeOH–water) and Fig. 6B (30:15:55% of ACN–MeOH–water). As can be seen in Fig. 6B, the retention times of estradiol and ketolevonorgestrel are 4.8 ($k' = 5.45$) and 4.6 ($k' = 5.19$) min, respectively. Their selectivity is 0.95 relative to estradiol. The experimental result is about the same as the predicted value. At the mobile phase composition of 30:15:55% of ACN–MeOH–water, the retention times of estradiol and ketolevonorges-

Table 5
Comparison of LSER coefficients

LSER coefficient[a]	At initial mobile phase composition[b]	At final mobile phase composition[c]	Change in coefficient[d]
m	1.963	1.914	−0.049(−2.5)
s	−0.221	−0.261	−0.04(−18)
a	−0.544	−0.432	0.112(+9.9)
b	−1.942	−1.837	0.105(+5.4)

[a] The LSER coefficients are computed based on Ref. [26].
[b] The initial mobile phase compositions are 40:60% of ACN–water.
[c] The final mobile phase compositions are 30:15:55% of ACN–MeOH–water. The LSER coefficients at this composition are used to predict the internal standard candidates.
[d] Change in coefficient. The values in the parentheses indicate the relative change to the initial values.

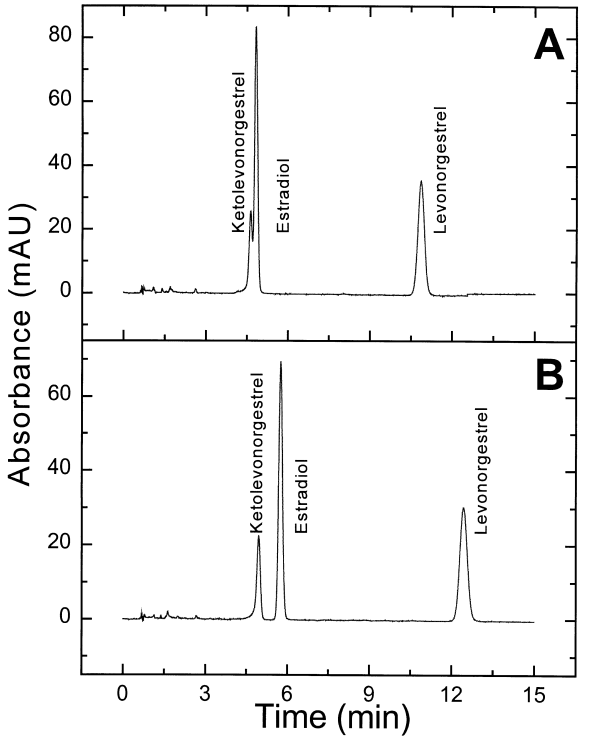

Fig. 6. Illustration of the chromatograms obtained for a patch sample consisting of estradiol, ketolevonorgestrel and levonorgestrel at 35:7:58% (A) and 30:15:55% (B) ACN–MeOH–water.

trel are 5.8 ($k' = 6.75$) and 5.0 ($k' = 5.67$) min, respectively (Fig. 6B). Their selectivity is 0.84, which is very consistent with the predicted result (0.88). It is noted in Fig. 6B that a sufficient resolution is obtained between ketolevonorgestrel and estradiol. Moreover, the selectivity of impurities relative to both estradiol and levonorgestrel at this mobile phase composition is evaluated, and no interference is observed.

It is noted in Figs. 3 and 6 that the retention time of levonorgestrel is shifted from 9 to 12.5 min. The shift in separation time is not significant. Because an adequate resolution is obtained and no further interference is observed, the mobile phase composition of 30:15:55% of ACN–MeOH–water is the final mobile phase composition used for the quantitation. The corresponding final LSER coefficients are also shown in Table 5. They are used to predict the internal standard candidates.

4.3. Selection of an internal standard compound

After having finalized the mobile phase composition, the next issue is to find a suitable internal standard for the separation. The approach taken in this work is to predict compounds that would elute at the position desired based on the ''open window'' in the chromatogram. The prediction is based on the relative retention [6].

4-Nitrotoluene is used as a reference solute to search for the internal standard. An examination of the chromatogram of a patch sample in Fig. 7A indicates that there is an ''open window'' around 10 min. If we select 10 min as the retention time of the internal standard, its selectivity relative to 4-nitro-

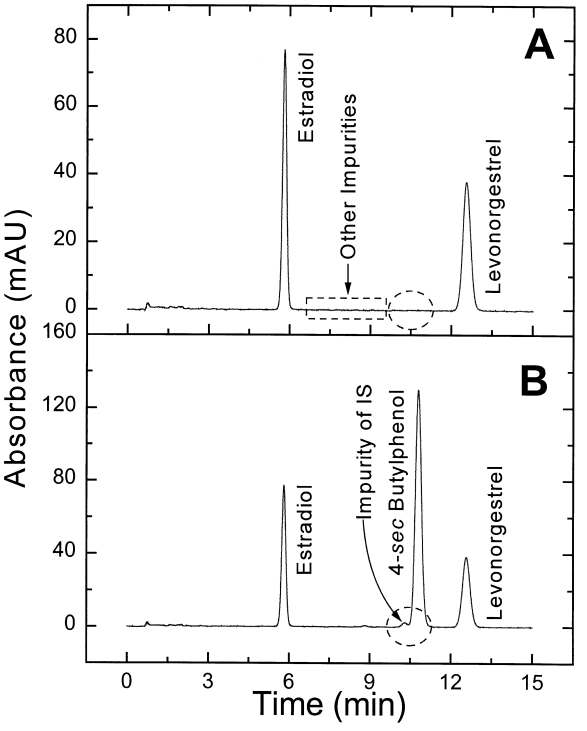

Fig. 7. Illustration of the chromatograms obtained for a patch sample of estradiol and levonorgestrel with and without internal standard 4-*sec*.-butylphenol at 30:15:55% ACN–MeOH–water. (A) The desired position for an internal standard (the dashed circle). The region between 6.5 and 9.5 min may not be used for the internal standard due to other potential interference. (B) The chromatogram with 4-*sec*.-butylphenol as the internal standard. The position of 4-*sec*.-butylphenol is within the desired range.

toluene is 1.73. This selectivity is the criterion that internal standard candidates should meet. Therefore, Eq. (3) together with the LSER coefficients in Table 5 is used to search for the compounds in a database (about 700 compounds) established previously [6,30]. The compounds selected are shown in Table 3 along with the retention times obtained experimentally. It is noted in Table 3 that 4-butylphenol is predicted to be the best match for the internal standard. However, we are not sure which isomer it represents. So both 4-*sec*.-butylphenol and 4-*tert*.-butylphenol are experimentally evaluated.

Based on the results in Table 3, 4-*sec*.-butylphenol has a retention time of ca. 10.3 min (the best match with a measured selectivity of 1.78). Moreover, this compound is stable and readily available with high purity. Accordingly, it was adopted as the internal standard for the method. 4-*tert*.-Butylphenol does not match the selectivity requirement. Fig. 7B shows the chromatogram obtained for a patch sample with the addition of 4-*sec*.-butylphenol.

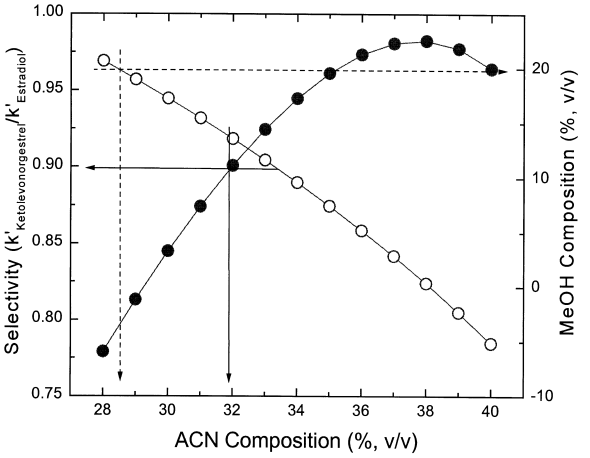

Fig. 8. Illustration of the change in the mobile phase composition and selectivity at a constant levonorgestrel retention time. The retention time of levonorgestrel is kept at 12.5 min. ACN concentration changes from 28 to 40%. Left plot (solid symbol), selectivity; right plot (open symbol), the corresponding MeOH concentration computed by Eq. (9).

4.4. Selectivity optimization by RSD

As mentioned early, RSM is used to compare the effectiveness and experimental requirement of the LSER approach for selectivity optimization. The two factors, five levels CCD allows us to evaluate the change in selectivity between ketolevonorgestrel and estradiol with the ternary mobile phase composition. The response data in Table 4 are fitted to Eq. (8) by Excel. The regression coefficients are shown in Table 6. It can be seen from Table 6 that the overall correlation is excellent for both levonorgestrel retention time and selectivity.

Table 6
Regression coefficients and statistics obtained for levonorgestrel retention time and selectivity

Coefficient	t_{Levo}	SD of t_{Levo}	α	SD of α
Y	123.362	9.051	−0.1057	0.1139
A	−4.434	0.407	0.0453	0.0051
B	−2.109	0.410	0.0148	0.0052
C	0.040	0.005	−0.0004	0.0001
D	0.004	0.009	−0.0005	0.0001
E	0.041	0.009	−0.0002	0.0001
R^2	0.99		0.99	
SE	1.29		0.016	

Based on the fitting results in Table 6, we can predict the retention and selectivity at any mobile phase composition within the ranges studied. To facilitate the comparison with the LSER result, we fix the levonorgestrel retention time to be 12.5 min (Fig. 6B, obtained at 30:15:55% of ACN–MeOH–water, the selectivity obtained at the mobile phase composition is 0.84). Then we compute the mobile phase compositions by Eq. (9) and the results of the calculation are shown in Fig. 8. It can be seen in Fig. 8 that, if the desired selectivity is $\leq$0.9, ACN concentration is $\leq$32%. Moreover, because the mobile phase range we studied is from 5 to 20% MeOH, ACN concentration should be $\geq$28.5%. Thus, if we select ACN concentration of 30%, the predicted MeOH concentration is 17% to provide a selectivity of about 0.85. This predicted selectivity is consistent with the measured (0.84) and LSER (0.88) results.

Overall, the selectivity predicted by both LSERs and RSM are almost identical; however, much more experiments are needed to obtain the response surface.

5. Conclusions

This paper shows the application of LSERs for selectivity optimization and prediction of internal standard candidates during RPLC method development. This approach provides a quantitative guidance for the mobile phase composition during selectivity optimization. The prediction of internal standard candidates proves accurate and efficient. The approach used in this study can be applied to other separations in RPLC method development. Compared to RSM, the LSER approach for selectivity optimization is more efficient (less experiments), and their accuracy is about the same.

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