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Optimization of throughput in semipreparative chiral liquid chromatography using stacked injection

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Abstract

An interesting mode of chromatography for preparation of pure enantiomers from pure samples is the method of stacked injection as a pseudocontinuous procedure. Maximum throughput and minimal production costs can be achieved by the use of total chiral column length in this mode of chromatography.

To maximize sample loading, often touching bands of the two enantiomers is automatically achieved. Conventional equations show direct correlation between touching-band loadability and the selectivity factor of two enantiomers. The important question for one who wants to obtain the highest throughput is "How to optimize different factors including selectivity, resolution, run time, and loading of the sample in order to save time without missing the touching-band resolution?" To answer this question, tramadol and propranolol were separated on cellulose 3,5-dimethyl phenyl carbamate, as two pure racemic mixtures with low and high solubilities in mobile phase, respectively. The mobile phase composition consisted of n-hexane solvent with alcohol modifier and diethylamine as the additive. A response surface methodology based on central composite design was used to optimize separation factors against the main responses. According to the stacked injection properties, two processes were investigated for maximizing throughput: one with a poorly soluble and another with a highly soluble racemic mixture. For each case, different optimization possibilities were inspected. It was revealed that resolution is a crucial response for separations of this kind. Peak area and run time are two critical parameters in optimization of stacked injection for binary mixtures which have low solubility in the mobile phase.

KEYWORDS

enantiopurification, method development, preparative chiral chromatography, propranolol, sample loading, stacked injection, throughput, tramadol

1 | INTRODUCTION

Direct resolution of chiral compounds using liquid chromatography (LC) has become the preferred tool for separation of enantiomers in pharmaceutical studies to provide each one with physiological, toxicological, and clinical evaluations, as stipulated by the US Food and Drug Administration (FDA). Extensive developments have been made in order to achieve efficient large-scale enantiopurification using preparative chiral LC.[1] Simulated moving bed (SMB) chromatography has been known as the most productive method in industrial-scale chiral chromatography, but these systems are more complex and require very special and expensive apparatus as well as the need for isotherm determination,

and hence considerable care may cause too much time for preparation. Although it is completely acceptable for industrial applications, in practical processes of research where time and sample size are limited (enantiomers at the range of grams), scientists prefer to use rapid batch chromatography in semipreparative scales.

An attractive practical method for preparation of pure components from binary mixtures by the use of a single column is to utilize the stacking mode for sample injection, making the total column length work, which eventuates in maximum throughput and consequently low production costs. To maximize sample loading, touching bands of the two enantiomers takes place automatically. Since in concentration overloading, throughput is determined by a selectivity factor (α), a high value of α is always recommended.[2] However, in practice, the solubility limitation in the mobile phase (MP) causes band touching of the peaks with volume overloading and this does not allow all the stationary phase to work, so that the column diameter determines throughput. Also, big band broadening causes a long delay between injections. Stacked injection does not require a big α, but needs baseline separation; therefore, optimizing the resolution (*Rs*) is more important than α for stacked injection. Since the MP composition in stacked injection has to be the same as that of the feed solvent to avoid baseline perturbation, another difficult task is optimization of the MP to keep the baseline separation alongside the dissolving power. This is the main aim of this work: to show that stacked injection could overcome low throughput due to the solubility limitation. While an MP providing sufficient *Rs* as well as high solubility of the enantiomers would be excellent for stacked injection on a single-batch column, usually low solubility of the enantiomers in MP convinces experts to change the MP or sample solvent, or to use another resolving method like crystallization. To solve this problem for stacked injection of poorly soluble enantiomers, a new method development is introduced according to the simultaneous optimization of MP and dissolving power. A well-known drug, tramadol, with low solubility in normal phase (NP) was used as a model enantiomer of this kind.

For highly soluble enantiomers in MP, usually chromatographists prefer to use nonlinear overloading instead of baseline resolution, but this is labor-intensive to optimize the recovery and purity at the same time, either experimentally or theoretically. Undoubtedly, stacked injection by touching-band overloading, which provides 100% recovery and purity, is simpler and more rapid for lab-scale production. Therefore, throughput of stacked injection for propranolol was investigated as a model of highly soluble enantiomers in MP.

To optimize a separation, we need to discover how different parameters affect the separation. Thus, the influence of the most effective factors on chiral separation of tramadol and propranolol had to be inspected. Chiral separation on a polar stationary phase with a nonpolar MP containing an organic modifier was ordered as NP chromatography. The effect of the MP composition on the retention in NP chromatography, operating under conditions of a linear isotherm, has been described using theoretical models of adsorption, developed in previous studies.[3-6] In these models, the adsorption process on a polar adsorbent surface was defined as a competition between the molecules of the solute and those of a modifier for adsorption sites. Despite some differences, all the models after simplifications led to the same simple equation describing the retention of the solute as a function of concentration of a strong solvent (the modifier):

$$\boldsymbol{k_i} = \boldsymbol{k}_{0,i}(\boldsymbol{C_{mod}})^{-\boldsymbol{m}_i} \tag{1}$$

where $\boldsymbol{k}_0$ is the retention factor of the solute in pure modifier, $\boldsymbol{C_{mod}}$ is the percentage of modifier, and $\boldsymbol{m}$is the empirical constant, which is determined by fitting to the set of experimental retention data acquired at different modifier contents in the MP. α (related to the two components) easily gains from the above equation:

$$\propto_{1,2} = \frac{\boldsymbol{k}_2}{\boldsymbol{k}_1} = \frac{\boldsymbol{k}_{0,2}(\boldsymbol{C_{mod}})^{-\boldsymbol{m}_2}}{\boldsymbol{k}_{0,1}(\boldsymbol{C_{mod}})^{-\boldsymbol{m}_1}} = \frac{\boldsymbol{k}_{0,2}}{\boldsymbol{k}_{0,1}}(\boldsymbol{C_{mod}})^{-\boldsymbol{m}_2+\boldsymbol{m}_1} \tag{2}$$

Once 1 and 2 are enantiomers, in a pure modifier it is acceptable to assume $\boldsymbol{k}_{0,1} \cong \boldsymbol{k}_{0,2}$ and $\boldsymbol{m}_1 > \boldsymbol{m}_2$, which results in

$$\propto_{1,2} = (\boldsymbol{C_{mod}})^{+\boldsymbol{m}} \tag{3}$$

This model shows a very straightforward linear positive relation between α and C_{mod}. It is a useful estimation but does not provide further information about the combination effects with other factors, such as flow rate and/or concentration of the additive. In some cases, this model is not complete at all to fulfill the entire requirements of stacked injection. Therefore, a central composite design (CCD) on surface response methodology including three factors of modifier and additive concentrations as well as the flow rate, with further analysis of variance (ANOVA) analysis (which easily allows modeling of the combined effects) was used for accurate investigations.[7]

Careful culling of the responses is probably the most important concern of this kind of modeling. Fortunately, there are well-known responses in chromatography to satisfy all the requirements such as *k*, α, *Rs*, run time (*RT*), peak area (*A*), etc.[8] Meanwhile, α is the most important response in preparative chromatography, but since stacked injection needs touching-band resolution, *Rs* might be more useful. The usefulness of α and/or *Rs* in chiral separation with stacked injection will be discussed further. To take the highest mass loading in the lowest band broadening, *A* and *RT* were

modeled for the three selected factors to be inserted in optimization.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Tramadol and propranolol were provided from TEMAD (Karaj, Iran). Ethanol and n-Hexane were of BP-grade, while di-ethylamine was of analytical-reagent grade quality; all were obtained from Chem-Lab (Zedelgem, Belgium). n-Hexane was double-distilled and the ethanol was filtered after drying with magnesium sulphate in order to be prepared for NP high-performance liquid chromatography (HPLC).

2.2 | HPLC

HPLC-U*V*/Vis analyses were performed on a Shimadzu LC-10ADvp system, equipped with solvent delivery systems (LC-10ADvp), SPD-10A U*V*/Visible dual detector, and LC Lab-Solution software (Shimadzu, Kyoto, Japan).

Tramadol and propranolol were separated on two analytical chiral columns (based on cellulose tris-3,5-dimethyl phenyl carbamate): Tramadol on Chiralcel OD-H (5 μm, 300 Å, from Chiral Technologies, West Chester, PA) and propranolol on a homemade column containing 20 wt% coating on triethoxy-3-amino-propylized silica gel (10 μm, 120 Å). CSP was prepared according to previous procedures.[9] Then 3 g of CSP was sonicated in a 30 mL mixture of paraffin/2-propanol/n-hexane (2:1:9) and poured into a 0.46 × 25 cm stainless steel HPLC column, preconnected to a reservoir. The column was packed by a Knauer Smartline Pneumatic Pump with 250-ml pump head (Berlin, Germany) supplying 2-propanol/n-hexane (1:10) at 4500 bar.

All HPLC analyses were carried out at ambient temperature for saturated and 100 ppm samples of tramadol and propranolol in MP, respectively. All saturated samples were filtered prior to injection.

According to our previous knowledge, MP compositions of n-hexane/ethanol/diethylamine and n-hexane/2-propanol/diethyl-amine were used for isocratic separation of tramadol and propranolol, respectively.[10,11] A UV wavelength of detector was adjusted to 271 nm for tramadol and 290 nm for propranolol.

2.3 | Software

The computer simulations were performed using Design-Expert 9.0.6 Trial (Stat-Ease, Minneapolis, MN) as the experimental design software. It offers a large number of different classes of design and a wide range of analytical and graphical techniques for model fitting and interpretation, while it is quite easy to run.

2.4 | Development of an experimental design model using CCD

Two-level factorial design (FD), full or fractional, is arguably the most widely used design in experimental investigations and is mainly used for the screening portion of experiments. Each CCD for n number of $x_1, \ldots, x_n$ coded factors is composed of three parts: a factorial (or cubic) design (FD), an axial, and a total of n_c runs at the center point of the experimental region. FD includes $n_{fact} = 2^n$ points with coordinates of $x_i = -1$ or $x_i = +1$, for $i = 1, \ldots, n$. Axial includes $n_{ax} = 2n$ points with all their coordinates null except for the one that is set equal to a certain value α (or $-\alpha$), which usually ranges from 1 to $n^{0.5}$. For center points there is $x_1 = x_2 = \ldots = x_n = 0$. In this study, two rotatable CCD with = 1.68 were used for optimization. Table 1 shows the levels of factors for the rotatable CCD and their notations related to tramadol and propranolol. Two levels (+1 and −1) of each factor were imported to the software, and the designs were constructed at five levels ($+\alpha$, +1, 0, −1, $-\alpha$) by 15 experimental runs for each of them. If FD is used considering five levels and three factors, 5^3, 125 experimental runs must be performed. Therefore, by applying the CCD against FD, the total required experimental runs were reduced by more than eight times for each optimization. Here, three effective factors of C_{mod}, additive percentage (C_{add}), and flow rate (F) were selected (Table 1).

k and Rs were calculated using the following well-known equations:

$$\boldsymbol{k} = (\boldsymbol{t}-\boldsymbol{t}_0)/\boldsymbol{t}_0 \tag{4}$$

$$\boldsymbol{Rs} = 1.18(\boldsymbol{t}_2-\boldsymbol{t}_1)/(\boldsymbol{w}_{0.5\boldsymbol{h}_2} + \boldsymbol{w}_{0.5\boldsymbol{h}_1}) \tag{5}$$

where $\boldsymbol{t}$ and $\boldsymbol{w}_{0.5\boldsymbol{h}}$ are the peak maxima time and the peak width at half height, respectively. For more investigation, two responses of RT and A which were effective on

TABLE 1 Factors and their levels for CCD

			Level				
	Factor	**Code**	**−α**	**−1**	**0**	**+1**	**+α**
T[a]	F (min/mL)	x_1	0.10	0.32	0.65	0.98	1.20
	C_{mod} [c] (%)	x_2	0.00	0.41	1.00	1.60	2.01
	C_{add} (%)	x_3	0.02	0.43	1.02	1.62	2.02
P[b]	F (min/mL)	x_1	0.50	0.60	0.75	0.90	1.00
	C_{mod} (%)	x_2	10.0	16.10	25	33.9	40.0
	C_{add} (%)	x_3	0.10	0.18	0.3	0.42	0.50

[a]T refers to tramadol.

[b]P refers to propranolol.

[c]Modifiers for T and P are ethanol and 2-propanol, respectively.

TABLE 2 Design matrix and the responses for central composite design (CCD) of T and P

Std	x_1 F (mL/min)		x_2 C_{mod} (%)		x_3 C_{add} (%)		y_1 k_1		y_2 k_2		y_2/y_1 α		y_3 Rs		y_4 RT (min)		y_5 $A(10^6$v/s)	
	T	P	T	P	T	P	T	P	T	P	T	P	T	P	T	P	T	P
1	0.32	0.60	0.41	16.08	0.43	0.18	1.52	1.34	1.85	2.80	1.22	1.68	2.21	1.34	25.0	28.0	6.385	5.267
2	0.98	0.90	0.41	16.08	0.43	0.18	2.16	1.65	2.73	2.91	1.26	1.69	1.18	1.65	9.1	21.0	4.307	2.822
3	0.32	0.60	1.60	33.92	0.43	0.18	0.78	0.79	0.93	1.42	1.19	1.47	1.10	0.79	17.8	15.5	15.29	6.322
4	0.98	0.90	1.60	33.92	0.43	0.18	0.91	0.87	1.03	1.46	1.13	1.46	0.90	0.87	6.0	11.5	3.093	2.843
5	0.32	0.60	0.41	16.08	1.62	0.42	1.90	1.57	2.23	2.67	1.18	1.81	1.38	1.57	27.2	29.0	11.35	5.400
6	0.98	0.90	0.41	16.08	1.62	0.42	1.95	1.65	2.40	2.75	1.23	1.79	1.53	1.65	9.2	21.0	2.767	2.8565
7	0.32	0.60	1.60	33.92	1.62	0.42	0.90	0.88	1.01	1.38	1.12	1.53	1.08	0.88	16.8	16.0	8.266	6.874
8	0.98	0.90	1.60	33.92	1.62	0.42	1.05	0.98	1.23	1.41	1.17	1.49	1.16	0.98	5.7	11.5	2.257	8.703
9	0.10	0.50	1.01	25.00	1.02	0.30	0.90	1.11	1.09	1.50	1.21	1.57	1.82	1.11	58.0	22.0	10.670	8.955
10	1.20	1.00	1.01	25.00	1.02	0.30	1.50	1.05	1.83	1.67	1.22	1.56	2.11	1.05	6.2	12.0	1.366	5.032
11	0.65	0.75	0.00	10.00	1.02	0.30	6.49	2.02	8.47	4.12	1.30	1.82	1.54	2.02	37.0	33.0	16.717	4.973
12	0.65	0.75	2.01	40.00	1.02	0.30	2.97	0.84	3.17	1.16	1.07	1.46	0.89	0.84	16.0	12.0	3.243	5.334
13	0.65	0.75	1.01	25.00	0.02	0.10	1.33	1.26	1.63	1.80	1.23	1.58	2.65	1.26	10.4	16.0	1.477	5.149
14	0.65	0.75	1.01	25.00	2.03	0.50	1.14	1.14	1.31	1.60	1.15	1.63	1.51	1.15	9.6	15.5	3.635	6.854
15	0.65	0.75	1.01	25.00	1.02	0.30	1.53	1.10	1.86	1.66	1.22	1.55	2.28	1.10	10.3	16.0	1.961	6.260

optimization of chromatography in stacked injection mode were included (Table 2).

3 | RESULTS AND DISCUSSION

3.1 | Modeling of k, α, and Rs

An ANOVA table was used to select a suitable response surface model, the significance of the model evaluation, and the model terms. Tables 3 and 4 show the ANOVA tables for CCD design matrix of the responses related to tramadol and propranolol, respectively. Quadratic and cubic response surface models were modified based on higher F and R values and lower *P*-value to fit the experimental data.

As predicted, the provided models for retention factors using ANOVA analysis showed both enantiomers of tramadol and both enantiomers of propranolol, following the same models due to similar interactions, of course in mirror orientation.

The retaining behavior of tramadol and propranolol enantiomers could be described using the following parametric eqs. 6 and 7, respectively:

$$k_T = \left(C - a_1 C_{mod} + a_2 C_{mod}^2 - a_3 C_{mod}^2 F^2 + a_4 F\right)^2 \quad (6)$$

$$k_P = \left(C' + a_1' C_{mod} + a_2' C_{mod}^2\right)^2 \quad (7)$$

As indicated, F has a significant effect on k_T but no effect on k_P. The best fitted model for α in both cases of tramadol (equation 8) and propranolol (equation 9) were found to be logit instead of power model. This clearly shows that there would be substitution for α model if we just rely on k models and/or without taking into account the other factors:

$$\ln\left[(\alpha_T - 1.07)/(1.30 - \alpha_T)\right] = C - a_1'' C_{mod}\left(1 + b F^2\right) \quad (8)$$

$$\ln(\alpha_P - 1.46/1.83 - \alpha_P) = C - a_2'' C_{mod} + a_2'' C_{mod}^2\left(1 - b' F + c' C_{add}\right) \quad (9)$$

These models clearly show that C_{add} has no effect on k and α, but a minimum on selectivity of propranolol enantiomers. The outstanding effect of C_{add} is observed on *Rs,* especially for tramadol (Tables 3 and 4). It affects the *Rs* by means of CSP endcapping results, in preventing an undesirable peak fronting and tailing, both of which can cause unresolved peaks.

The investigated models revealed that, in some cases, optimization of α without *Rs* is not sufficient to achieve the maximum productivity in chromatography. However, C_{mod} is the most effective parameter, either alone or in combination with the two other factors, and also in fast estimations it is the most convenient choice. A significant effect of C_{mod} often overcomes the influence of other factors in the case of k, but not necessarily in the cases of *Rs*, *RT,* and sample solubility.

3.2 | Optimization procedure for throughput

Throughput and productivity are two useful parameters for evaluation of production units in chromatography.

TABLE 3 Analysis of variance (ANOVA) table of response surface model for k_1, k_2, α, Rs and A for tramadol enantioseparation

	Source	SS	Df	Mean square	F value	*P*-value Prob > F	
$(k_1)^{0.5}$	Model	1.88	4	0.47	64.30	< 0.0001	Significant
	x_1	2.43	4	0.61	129.82	< 0.0001	
	x_2	0.055	1	0.0550	11.77	0.0064	
	x_2^2	0.68	1	0.6800	145.52	< 0.0001	
	$x_1^2x_2^2$	1.46	1	1.4600	311.06	< 0.0001	
	Residual	0.38	1	0.3800	81.30	< 0.0001	
	Cor Total	0.047	10	0.0047			
	Actual equation			$2.107 + 0.303\ x_1 - 2.264\ x_2 + 0.973\ x_2^2 - 0.069\ x_1^2x_2^2$			
$(k_2)^{0.5}$	Model	2.43	4	0.6100	66.33	< 0.0001	Significant
	x_1	0.10	1	0.1000	11.01	0.0078	
	x_2	0.80	1	0.8000	87.45	< 0.0001	
	x_2^2	1.35	1	1.3500	147.48	< 0.0001	
	$x_1^2x_2^2$	0.30	1	0.3000	32.49	0.0002	
	Residual	0.091	10	0.9140			
	Cor Total	2.52	14				
	Actual equation			$2.359 + 0.356\ x_1 - 2.546\ x_2 + 1.069\ x_2^2 - 0.081\ x_1^2x_2^2$			
Logit(α) = ln[(α-1.07)/(1.30-α)]	Model	141.63	4	35.410	118.44	< 0.0001	Significant
	x_2	131.87	1	131.87	441.09	< 0.0001	
	x_3	1.42	1	1.4200	4.75	0.0543	
	x_2^2	4.54	1	4.5400	15.17	0.0030	
	$x_1^2x_2$	56.75	1	56.750	189.82	< 0.0001	
	Residual	2.99	10	0.3000			
	Cor Total	144.62	14				
	Actual equation			$3.330 - 0.791\ x_2 - 0.542\ x_3 - 1.718\ x_2^2 + 0.425\ x_1^2x_2$			
$(Rs)^{-2}$	Model	1.85	8	0.2300	100.16	< 0.0001	Significant
	x_2	0.76	1	0.7600	331.03	< 0.0001	
	x_3	0.044	1	0.0440	19.05	0.0047	
	x_1x_3	0.16	1	0.1600	70.07	0.0002	
	x_2x_3	0.032	1	0.0320	13.95	0.0097	
	x_2^2	0.49	1	0.4900	212.00	< 0.0001	
	$x_1x_2^2$	0.065	1	0.0650	28.18	0.0018	
	$x_3x_2^2$	0.068	1	0.0680	29.26	0.0016	
	$x_1^2x_2^2$	0.19	1	0.1900	83.74	< 0.0001	
	Residual	0.014	6	0.2308			
	Cor Total	1.86	14				
	Actual equation	$0.987 - 2.159\ x_2 - 0.245\ x_3 - 0.171\ x_1x_3 + 1.182\ x_2x_3 + 1.354\ x_2^2 - 0.180\ x_1x_2^2 - 0.677\ x_3x_2^2 + 0.262\ x_1^2x_2^2$					
Logit (A) = ln[(A - 1.36)/(16.72 - A)]	Model	168.7	4	42.18	33.31	< 0.0001	Significant
	x_2	54.9	1	54.88	43.34	< 0.0001	
	x_2^2	66.3	1	66.34	52.40	< 0.0001	
	$x_2x_3^2$	35.0	1	34.95	27.61	0.0004	
	x_1^3	47.4	1	47.35	37.40	< 0.0001	
	Residual	12.7	10	1.27			
	Cor Total	181.4	14				
	Actual equation	$6.61249 - 14.17954\ x_2 + 6.00490\ x_2^2 + 0.05828\ x_2x_3^2 - 3.44755\ x_1^3$					

Throughput is the amount of purified component(s) per time united, and productivity is throughput per volume united of the column bed.[12] Since this discussion excluded the scale-up and included a single column, throughput was studied as the more convenient parameter instead of productivity.

According to the solubility of a racemate in MP, two different procedures were reduced for modeling of tramadol and propranolol as poorly and highly soluble enantiomers, respectively:

(i) Saturated samples of tramadol in MP were injected for modeling. This procedure takes into account the solubility of enantiomers in MP to find the optimum separation along maximum concentration loading. A calibration curve (Figure 1) and a further modeling for A (Table 3) were required for accurate detection of solubilities and their effects.

(ii) Another procedure was performed on propranolol by injection of 100 ppm of the same concentrated samples in each requested MP. This procedure provides more accurate models for Rs, but needs further optimization on mass loading as suggested solutions by the software.

It has to be mentioned that k and α values were independent of injected concentration, because all runs were carried out in a linear region of the adsorption isotherms. Furthermore, the retention time of the peak that is used to calculate k and α is also independent of injected concentrations.

TABLE 4 Analysis of variance (ANOVA) table of response surface model for k_1, k_2, α, and Rs for propranolol enantioseparation

	Source	SS	Df	Mean square	F value	*P*-value Prob > F	
$(k_1)^{0.5}$	Model	0.76	2	0.3800	226.86	< 0.0001	Significant
	x_2	0.68	1	0.6800	404.72	< 0.0001	
	x_2^2	0.082	1	0.0820	49.00	< 0.0001	
	Residual	0.020	12	0.1674			
	Cor Total	0.78	14				
	Actual equation	$2.808–0.091\ x_2 + 0.001\ x_2^2$					
$(k_2)^{0.5}$	Model	1.71	2	0.8500	358.40	< 0.0001	Significant
	x_2	1.53	1	1.5300	642.99	< 0.0001	
	x_2^2	0.18	1	0.1800	73.82	< 0.0001	
	Residual	0.029	12	0.2383			
	Cor Total	1.74	14				
	Actual equation	$3.932–0.139\ x_2 + 0.002\ x_2^2$					
Logit(α) = ln[(α-1.46)/(1.83 - α)]	Model	89.30	4	22.32	124.99	< 0.0001	Significant
	x_2	77.14	1	77.14	431.89	< 0.0001	
	x_2^2	1.56	1	1.560	8.73	0.0144	
	$x_1x_2^2$	1.12	1	1.120	6.29	0.0310	
	$x_3x_2^2$	9.47	1	9.470	53.04	< 0.0001	
	Residual	1.79	10	0.180			
	Cor Total	91.09	14				
	Actual equation	$8.689–0.497\ x_2 + 0.004\ x_2^2–0.003\ x_1x_2^2 + 0.007\ x_3x_2^2$					
$(Rs)^{0.5}$	Model	0.340	5	0.0690	113.16	< 0.0001	Significant
	x_2	0.310	1	0.3100	513.71	< 0.0001	
	x_2^2	0.019	1	0.0190	30.92	0.0004	
	x_3^2	0.003	1	0.0031	5.17	0.0490	
	$x_1x_2^2$	0.008	1	0.0080	13.19	0.0055	
	$x_3x_2^2$	0.005	1	0.0048	7.95	0.0201	
	Residual	0.005	9	0.0006			
	Cor Total	0.350	14				
	Actual equation	$1.762–0.041\ x_2 + 0.0004\ x_2^2 + 0.159\ x_3^2 + 0.0001\ x_1x_2^2–0.00002\ x_3x_2^2$					

3.3 | Poorly soluble compound

To maximize α, optimization of the model was carried out in the range of 0 to 2% for both C_{mod} and C_{add}, and 0.1 to 1.2 mL.min^{-1} for F. All of the solutions suggested 1.3 for α at different values of F and RT, from 0.1 to 0.7 mL.min^{-1} and 20 to 44 min, respectively. Initially, the presented solutions seemed to be adequate, but more investigation revealed that in most of the solutions, the value of Rs is less than1.5, which causes them to be useless for the stacked injection required for touching-band separation. It clearly demonstrates the inadequacy of the optimization of α without considering Rs in this mode of preparative chromatography. Since α does not provide information on the quality of separations, Rs was applied as a more accurate factor. Inevitably, maximizing the Rs would cause obtaining acceptable values for α, while this is not true in the opposite way, which is another reason for paying more attention to Rs in optimizations of this kind. If the peaks are perfectly symmetric, the valley between the peaks should just touch the baseline when $Rs = 1.5$. This is exactly what the stacked injection with band touching requires. Under this condition, 100% recovery and purification are achieved and the amount of purified enantiomers is equal to the loaded mass.

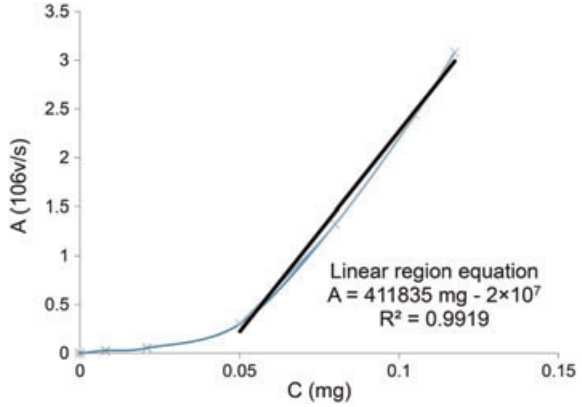

FIGURE 1 Tramadol calibration curve

The highest throughput points in three possible modes of optimization including minimizing RT, maximizing A, and optimizing between the maximum A and the minimum RT, were calculated. Then throughput graphs were plotted against Rs (Figure 2). These plots show important results about how to achieve the maximum throughput. When focusing on maximizing A, one has to use the most power of dissolving composition for MP, but not composition with maximum Rs. According to the graphs, this method provides lower throughputs.

When keeping a focus on minimizing RT, although the dissolving power is lost, the lower operating time compensates for the lower mass injection in final throughput. For more difficult separations without baseline resolution, more attention has to be paid to simultaneous optimization of a desirable recovery, purity and throughput, which of course,

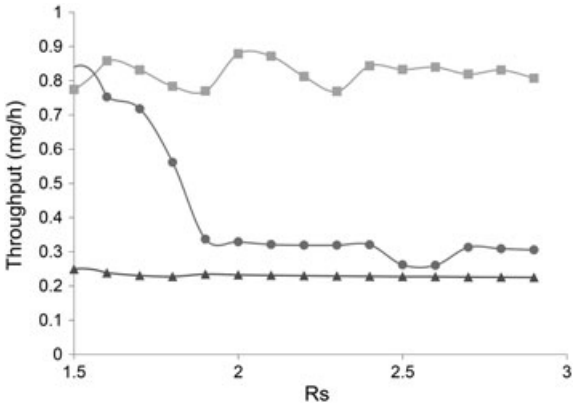

FIGURE 2 Throughput variation against *Rs* for three optimization mode: *RT* minimizing (squares), *A* maximizing (triangles), and optimizing between minimum *RT* and maximimum *A* (circles)

is not about the present work concerning stacked injection with touching-band loading.

Optimization between maximum *A* and minimum *RT* does not provide higher throughputs than that in the case of just minimizing *RT*, and is less reliable for higher *Rs*. The trend toward bigger *Rs* shifts throughputs from close to minimum *RT* results to those of maximum *A*.

According to the optimization for minimum *RT* in $Rs = 1.5$, $C_{mod} = 1.4$, $C_{add} = 2.0$ and $F = 1.2$ mL.min^{-1} was suggested by the model. This MP composition provides 4300 ppm of the sample. The accuracy of the model was checked by performing a single run and, subsequently, the stacked injection was carried out (Figure 3).

The throughput value for this optimization in batch to batch mode is 1.78 mL.min^{-1} with 2.9 min for injection intervals according to $\Delta t = t_2 - t_0 = (4.5 - 1.6)$ min. The stacked injection mode at 1-min intervals increased the throughput from 1.78 to 5.16 mg.h^{-1}, which is three times more.

For comparison of stacked injection with the overloading condition, *Rs* was maximized in minimum *RT*. $Rs = 3.1$ in $C_{mod} = 1.18$, $C_{add} = 2.00$ and $F = 1.2$ mL.min^{-1} was suggested by the model. A saturation limit for this MP composition was 3200 ppm. To enhance the dissolving power, ethanol was added so that MP would not spoil the touching-band resolution (Figure 4). If C_{mod} in the sample becomes more than that in MP, this can disrupt the baseline of the chromatogram.

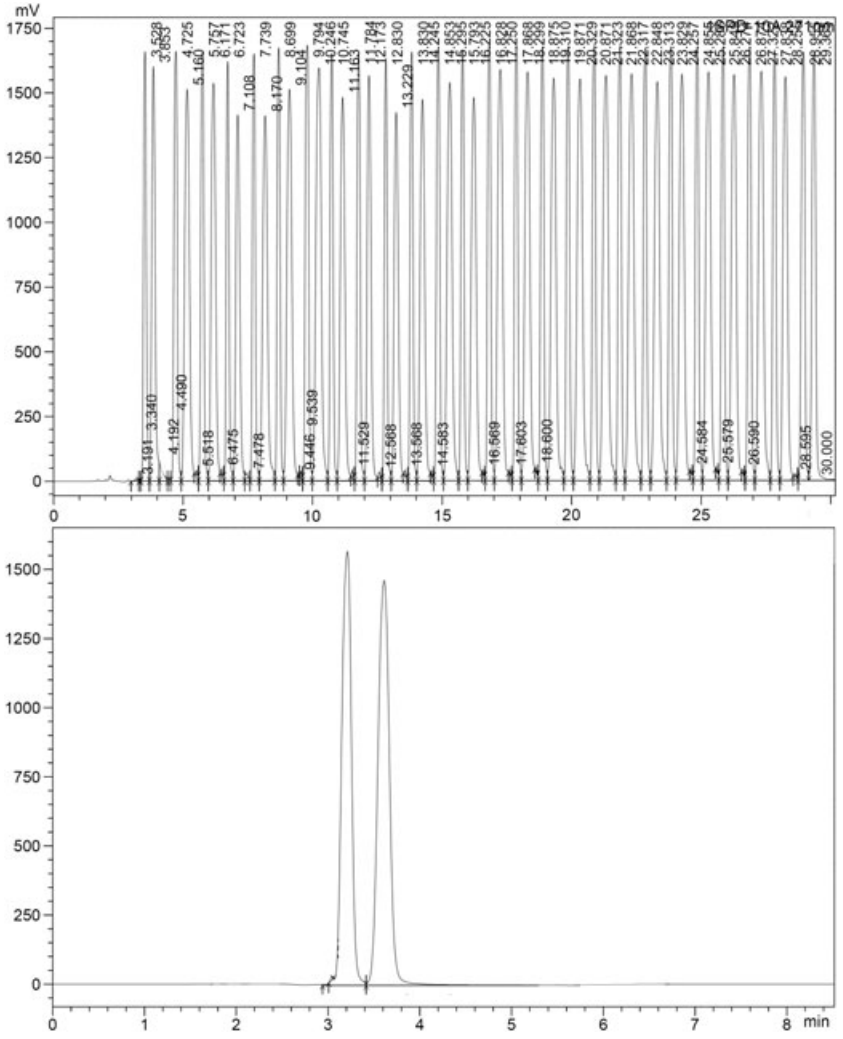

FIGURE 3 Chromatograms for 4300 ppm of tramadol (down) and its stacked injection (up) in $F = 1.2$ mL.Min^{-1} and MP composition of 1.4% ethanol and 2.0% DEA in n-hexane related to minimizing *RT* in $Rs = 1.5$ (sample solvent was identical to the MP)

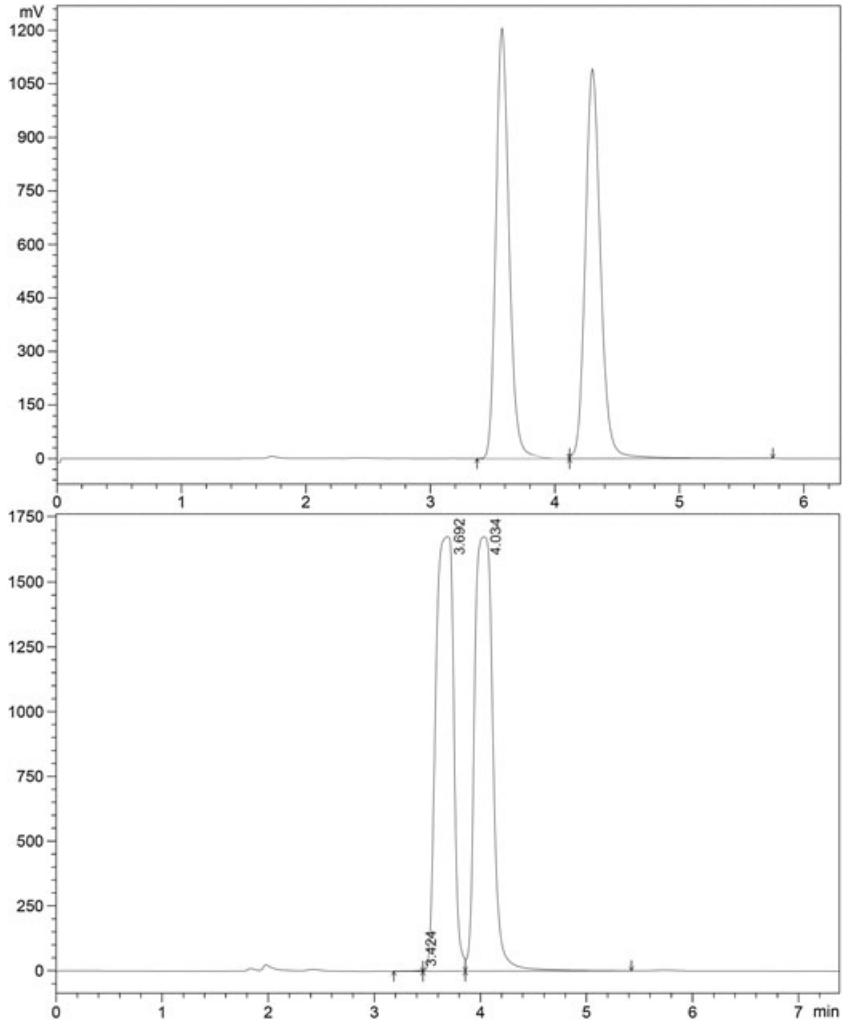

FIGURE 4 Chromatograms for 3200 ppm of tramadol dissolved in MP (up), and 12,000 ppm of tramadol dissolved in 2.00% ethanol and 2.00% DEA (down) related to maximizing *Rs* in minimum *RT* ($F = 1.2$ mL. Min^{-1} and MP composition was 1.18% ethanol and 2.00% DEA in n-hexane)

Of course, there are examples in which the baseline remains intact in high injection volumes of different solvents; but as a general rule, it is not recommended to use this kind of sampling for a stacked injection. The throughput value for the overloaded mode was calculated to be 10 $mg.h^{-1}$ with 2.4 min gap between successive injections.

3.4 | Highly soluble compound

Optimization by maximizing α brought 40% of *Rs* under 1.5. When maximization of α was coupled to minimizing *RT*, it pulled down 50% of *Rs* under 1.5; therefore, optimization has to be performed with *Rs* again.

Since modelings were performed at concentrations of 100 ppm, which is generally a low concentration for preparative targets, it is preferred to use a higher limitation for *Rs* instead of 1.5, to provide enough capacity for larger mass loadings. The limit was adjusted on $Rs = 1.8$. Because *Rs* and *RT* go against each other, minimizing *RT* pulls down all *Rs* to its lower limit of 1.8; therefore, inputting the correct limit for *Rs* is quite important. The best solution at this level was 40% C_{mod}, 0.6% C_{add}, and $F = 1$ $mL.min^{-1}$. As mentioned earlier, in this procedure the practical tests for loading have to be performed. The sample concentration for injection was increased to two levels from 100 to 1000 and from 1000 to 3000 ppm, where *Rs* was still satisfactory for baseline resolution (Figure 5).

A comparison between the stacked and overloaded injections exhibited approximately 2 times more throughput for the overloaded mode. Although an overloaded injection provided more throughput, the optimization mode for stacked injection is more reliable as a systematic process. Also, this process could be very beneficial in the optimization of chiral SMB units, where continuous feeding of the sample in MP takes place.

This condition containing real touching-band resolution was used for stacked injection of propranolol (Figure 6). The throughput value for this optimization in stacked injection mode was 1.03 $mg.h^{-1}$ with 3.5-min intervals.

In the following, *Rs* was just maximized without an up limitation, which resulted in $C_{mod} = 10\%$, $C_{add} = 0.6\%$, and $F = 1$ $mL.min^{-1}$ with $Rs = 3.44$ and $\alpha = 1.83$. High solubility of propranolol permitted dissolution of 20,000 ppm sample in MP for injection (Figure 7).

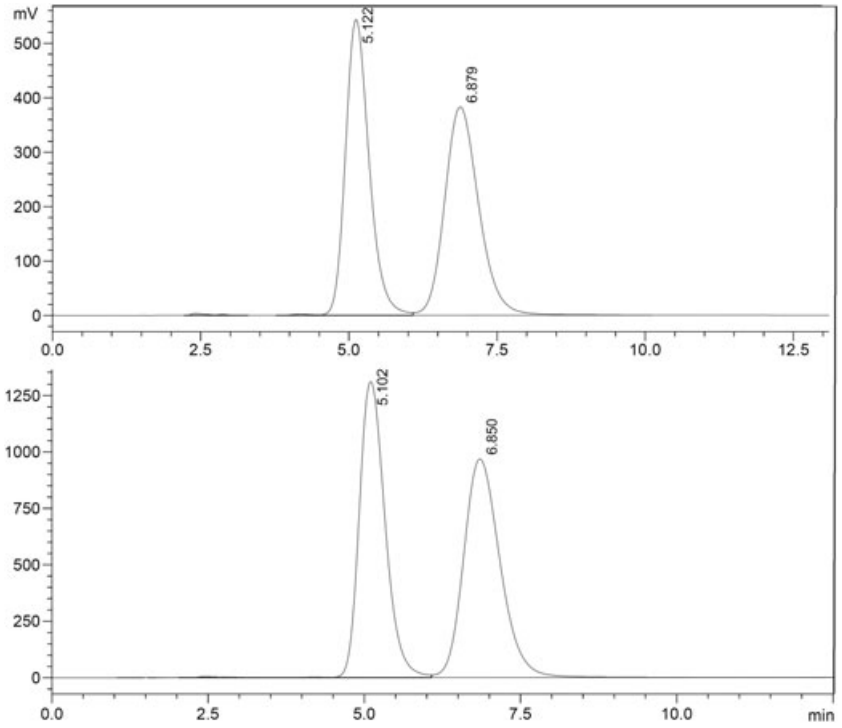

FIGURE 5 Chromatograms of 1000 ppm (up) and 3000 ppm (down) of propranolol in $F = 1$ mL.Min^{-1} and MP composition of 40% 2-propanol, 0.6% DEA, related to minimizing *RT* in $Rs \geq 1.7$ (sample solvent was identical to the MP)

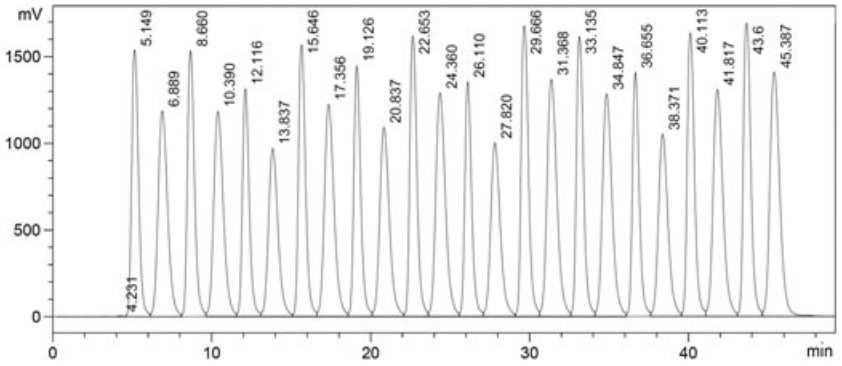

FIGURE 6 Chromatograms for 3000 ppm of propranolol in stacked injection mode ($F = 1$ mL.Min^{-1} and MP composition was 40% 2-propanol, 0.6% DEA in n-hexane; sample solvent was identical to the MP)

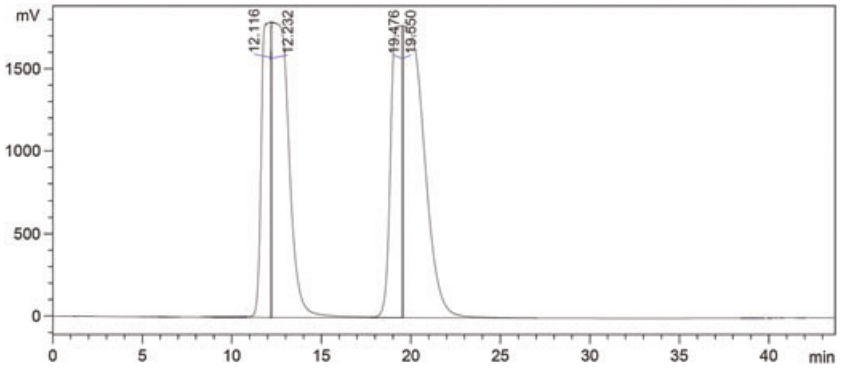

FIGURE 7 Overloaded chromatogram for 20,000 ppm of propranolol in $F = 1.0$ mL. Min^{-1} and MP composition of 10% ethanol and 0.60% DEA in n-hexane, related to maximizing *Rs* (sample solvent was identical to the MP)

The throughput value of this optimization in stacked injection mode (with 12-min intervals) was calculated to be 2.00 mg.h^{-1}. This clearly shows that for highly soluble samples in MP, stacked injection in maximum *Rs* provides higher throughput, even if it causes larger *RT* and/or is not a touching band ($Rs > 1.5$). The latter results in extra time between the peaks, which decreases throughput. Therefore, for samples of this kind, the first effort would be maximizing *Rs*.

Samples with high solubility in MP and satisfactory *Rs* are considered the best cases for stacked injection mode, hence prescreening the dissolving power of MP prior to starting chiral chromatography would be of special importance.

4 | CONCLUSION

Stacked injection in LC has some limitations, such as requiring the touching-band resolution of components in a sample mixture, high dissolution of sample in MP or any other solvent that does not disturb the baseline, and operating in isocratic mode. However, in some cases, particularly for purification of enantiomers, the stacked injection far surpasses batch chromatography and even other continuous methods. Preparative chiral chromatography is usually performed in NP in isocratic mode, where there are just two enantiomers having close retention times. Therefore, chiral chromatography often fulfils all prerequisites of stacked injection. The results of this study revealed that stacked injection presents very good productivity for poorly soluble tramadol, and the best one for highly soluble propranolol, rather than overloading with different solvents from MP in a batch chromatography of enantiomers.

For the first time, a modeling method via injection of saturated samples was optimized to overcome the limitation of the stacked injection for poorly soluble samples in MP. Providing close productivity to the overloaded condition for a difficult case such as tramadol shows the success of the introduced modeling approach. Although preparative resolution of tramadol by chiral chromatography has not been reported, the present procedure offers highly pure tramadol or any other enantiomer with low solubility in MP, with acceptable throughput.

Modeling of chiral separation for propranolol as a case with high solubility in MP demonstrated the power of stacked injection even under suboptimal conditions (not the highest possible concentration). Very pure propranolol or any other enantiomer with high solubility in MP is quickly producible with 100% recovery, just by maximizing *Rs*, and then increasing the loading to touching band or less.

$Rs \geq 1.5$ was respected in both cases to assure complete purification and recovery, but if *Rs* is not sufficient for baseline resolution ($Rs < 1.5$), further optimization between recovery, purification, and throughput has to be performed in order to allow the making correct decision.

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