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Chromatographic assay of pharmaceutical compounds under column overloading

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Abstract

A HPLC assay method utilizing overloaded chromatography and dual-wavelength detection was developed for a pharmaceutical formulation containing an antibacterial (clotrimazole) and a steroid (mometasone furoate) at widely different concentrations. In order to meet the limit of quantitation (LOQ) objective of not less than 0.05% of assay concentration simultaneously for both actives in the HPLC assay method, the assay concentration of the antibacterial falls into the non-linear range of its equilibrium isotherm, but still in the linear dynamic range of an ultraviolet detector. Although the analytical column is overloaded with the antibacterial and a non-symmetric elution peak is obtained, the HPLC assay method exhibits good linearity, recovery and reproducibility.

Keywords: Column overloading; Pharmaceuticals

1. Introduction

Nonlinear chromatography, which studies the concentration dependence of an elution peak, has been the topic of intense activity in the community of preparative chromatography during the past 10 years. Its fundamental concepts of non-linear chromatography form the basis for preparative chromatography in the laboratory, pilot and process scales [1]. The goal of preparative chromatography is to obtain purified chemical compounds. In order to maximize production rate, minimize solvent consumption and reduce cost, a preparative separation column is usually overloaded as much as possible to benefit from the non-linearity of a thermodynamic isotherm. On the other hand, the goal of analytical chromatography is to accurately and precisely quantitate chemical compounds. Therefore, symmetric Gaussian elution peaks are favored and injection concentrations are kept low to prevent overloading of the analytical column. This is usually not a problem for a drug formulation containing a single active, but in a multi-active formulation where the label strengths of the actives vary greatly, the drug compound with a high label strength is likely to overload the analytical column and to elute as a non-symmetric peak. If the non-symmetric peak can be effectively used to assay the overloaded solute and to achieve a limit of quantitation (LOQ) of 0.05% for both actives, a single high-performance liquid chromatography (HPLC) method can be developed that can assay both drug actives simultaneously, thus eliminating the need to use two HPLC methods. This increases efficiency, which is especially important for quality

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control operation in the pharmaceutical industry. Wilson et al. pointed out the possibility of using overloaded chromatography in drug assay [2], however, few if any examples are cited in the literature. In this paper, we discuss column overloading from the thermodynamic aspect and compare a chromatographic assay based on a symmetric Gaussian elution peak with one based on a non-symmetric elution peak. The sample used in this paper is a pharmaceutical formulation which contains two drug actives; one an antibacterial (clotrimazole) and the other a steroid (mometasone furoate). The label strength of the antibacterial is 10-times higher than that of the steroid. To achieve an LOQ of 0.05%, the assay elution concentration of the antibacterial falls in the non-linear range of its equilibrium isotherm and is eluted as a non-symmetric peak. The assay elution concentration of the steroid is in the linear range of its equilibrium isotherm and is eluted as a symmetric peak (Fig. 1). This paper demonstrates that under the condition of column overloading, and at the same time achieving an LOQ of 0.05% for both actives, acceptable linearity, recovery and reproducibility are obtained for the simultaneous assay of both compounds.

2. Theoretical background

2.1. Measurement of thermodynamic isotherm

Several methods can be used to measure a single component isotherm. They include frontal analysis (FA), frontal analysis by characteristic point (FACP), elution by characteristic points (ECP), pulse methods, retention time method, computation of elution profiles (CEP) method and static method [1]. In this paper, the method of retention time (RT) is used because all the required data is provided by the HPLC assay procedure. The RT method requires only two injections. One is a linear injection or a sample pulse, where the elution concentration of the solute is in the linear range of its isotherm and gives symmetric Gaussian elution profile. The other is an overloaded injection, where the elution concentration of the solute is in the non-linear range of its isotherm and results in a profile resembling a triangle with a sharp, nearly vertical front (Fig. 3 and Fig. 5). This method is simple and valid for a compound which follows the pattern of a Langmuir isotherm (Eq. (1)) in adsorption and desorption between the stationary phase and the mobile phase:

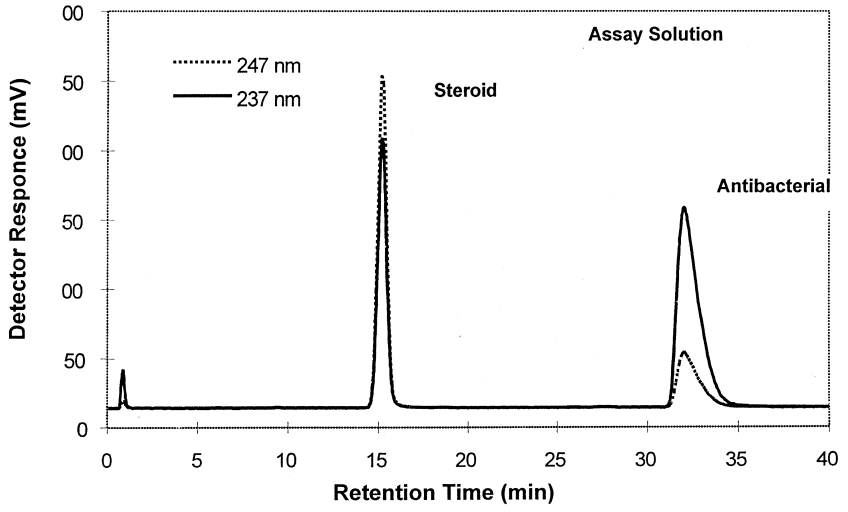

Fig. 1. Overlaid elution profiles of the assay solution containing both the steroid (3.12 μg) and the antibacterial (31.2 μg) under two different wavelengths, 247 nm (dashed line) and 237 nm (solid line). Experimental conditions: column: Beckman Ultrasphere 3 μm C_{18} 7.5×4.6 mm, temperature: 36°C, mobile phase: 10 m*M* sodium phosphate buffer, pH 6.5–water (41:59), injection volume: 50 μl, chromatograph: Waters 6000; detector: Thermoseparation Spectra SYSTEM UV 2000 dual-wavelength, 10 μl cell, 10 mm path length, 237 nm and 247 nm are utilized for the antibacterial and the steroid, respectively.

$$Q = \frac{aC}{1 + bC} \quad (1)$$

In Eq. (1), a and b are Langmuir coefficients, C is the concentration of the elute in the mobile phase, Q is the concentration of the elute in the stationary phase in equilibrium with the concentration C. Coefficient a can be obtained from the following equation:

$$a = \frac{t_{R,0} - t_0}{Ft_0} \quad (2)$$

where $t_{R.0}$ is the retention time of the linear injection, F is the phase ratio of a column, and t_0 is the void volume of the column. Langmuir coefficient b can be obtained from Eq. (3):

$$b = \frac{L_f F_v (t_{R,0} - t_0)}{n_m} \quad (3)$$

where F_v is the volumetric flow-rate of the mobile phase, n_m is the amount of elute injected and L_f is the loading factor. The loading factor corresponds to the amount of sample injected and can be derived from the retention time of the sharp front (t_f) of the overloaded elution profile:

$$L_f = \left[1 - \left(\frac{t_f - t_0 - t_p}{t_{R,0} - t_0} \right)^{1/2} \right]^2 \quad (4)$$

where t_p is the duration time of an injection [3].

3. Experimental

3.1. Apparatus

The following equipment was used: a Waters 6000 chromatograph (Milford, MA, USA); a Thermoseparation (Riviera Beach, FL, USA) Spectra SYSTEM UV 2000 dual-wavelength detector with 10 μl cell, 10 mm path length, 237 nm for the antibacterial and 247 nm for the steroid; a Beckman (Fullerton, CA, USA) Ultrasphere 3 μm C_{18} 7.5 cm×4.6 mm column. Other conditions were temperature: 36°C; mobile phase: 10 m*M* sodium phosphate buffer, pH 6.5–methanol (41:59).

3.2. Chemicals and reagents

Clotrimazole and mometasone furoate were provided by Schering-Plough (Kenilworth, NJ, USA). Methanol (HPLC grade) and sodium phosphate were obtained from Fischer Scientific (Fair Lawn, NJ, USA). Water (HPLC grade) was obtained using Milli-Q Water System.

4. Results and discussion

4.1. Equilibrium isotherm for the antibacterial

The resolution injection (Fig. 2) and the assay injection (Fig. 1) in the developed HPLC assay procedure are used, respectively, as the linear injection and the overloading injection discussed earlier for the RT method of measuring component isotherm. The injection amount of the antibacterial in the resolution injection is 10-times less than that in the assay injection. Fig. 3 shows an overlaid elution profile of the two injections for the antibacterial. From the retention times of these two injections, the Langmuir isotherm coefficients (see Table 1) a and b are obtained, and the isotherm for the antibacterial (Fig. 4) is plotted using Eq. (1).

4.2. Elution profile and thermodynamic isotherm

The dependence of the chromatographic elution profile of the antibacterial on its thermodynamic isotherm is demonstrated with seven standard solutions containing both the antibacterial and the steroid at concentrations representing 8%, 25%, 50%, 75%, 100%, 125% and 150% of label strengths. Equal volumes of the sample solutions were chromatographed and the resulting elution profiles are shown in Fig. 5.

The results demonstrate that the retention time of the steroid is constant and its elution profile remains symmetric as the injection concentration increases. In comparison, the retention time of the antibacterial decreases and its elution profile becomes less and less symmetric, until it finally resembles a triangle having a sharp front. The symmetric elution profile for the steroid indicates that its elution concentration is within the linear range of its isotherm. For the

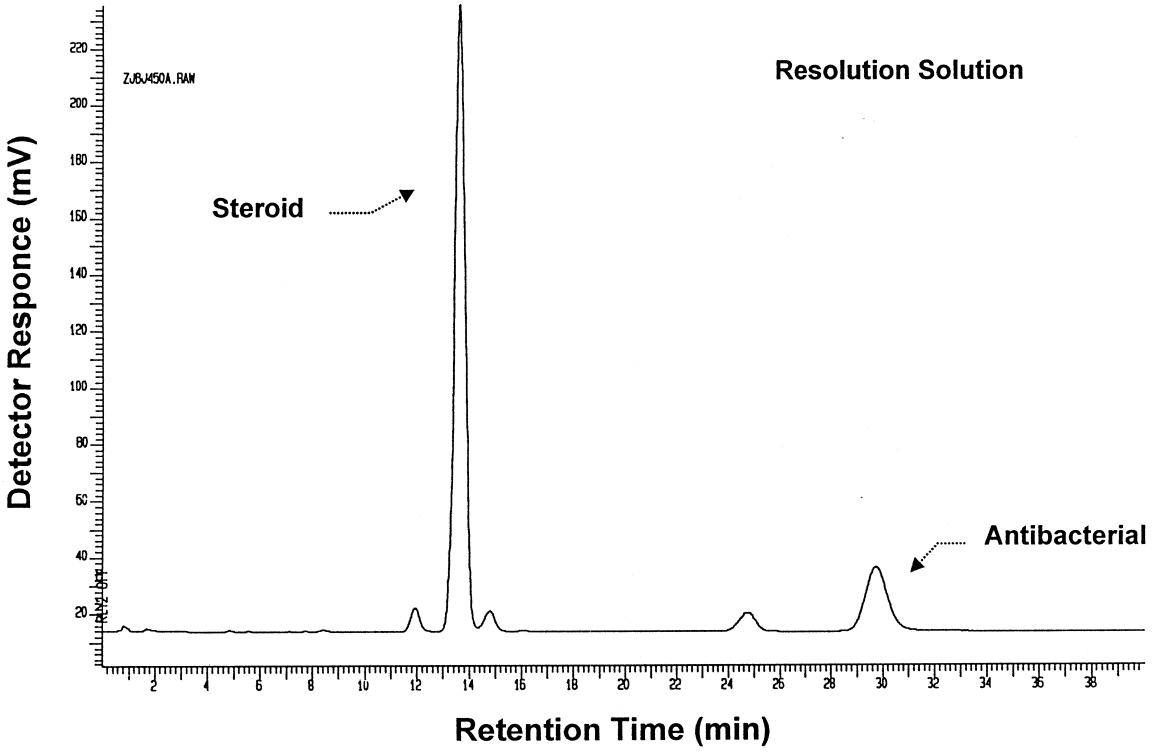

Fig. 2. Elution profiles of the resolution solution which contains the steroid (3.12 μg), the antibacterial (3 μg) and some related compounds. Experimental conditions as in Fig. 1.

antibacterial as shown in Fig. 6, the elution profile is symmetric if the elution concentration is in the linear range of its isotherm, and the elution profile is non-symmetric if the elution concentration is in the non-linear range of its isotherm.

It should be noted as illustrated in Fig. 6, that although most of the elution concentrations of the antibacterial are in the non-linear range of its equilibrium isotherm, they are all within the linear dynamic range of the UV detector.

4.3. Limit of quantitation and its impact on the HPLC assay method

In many pharmaceutical applications, one tries to achieve a signal-to-noise ratio (S/N) of not less than 10 with an LOQ solution that is not less than 0.05% of its assay concentration. For a single active formulation, it is possible to achieve an assay concentration which is low enough to be in the linear range of its isotherm and high enough so that its 0.05% LOQ solution meets a S/N of not less than 10. For the subject multi-active formulation that we have, this becomes difficult or even impossible, especially since the label strengths of the actives vary greatly, and they have very different detector response factors. In order to have the LOQ at 0.05% level for both drug actives in a simultaneous determination, the assay concentration of the antibacterial falls in the non-linear range of its isotherm and it overloads the analytical column. We can not decrease the injection amount to avoid column overloading because any reduction correspondingly reduces the amount of steroid injected, and thus, the steroid falls short of the required S/N ratio. We chose to keep the assay concentration of the antibacterial in the non-

Table 1
Langmuir coefficients of the antibacterial

a	61
b	2.52 (ml/mg)

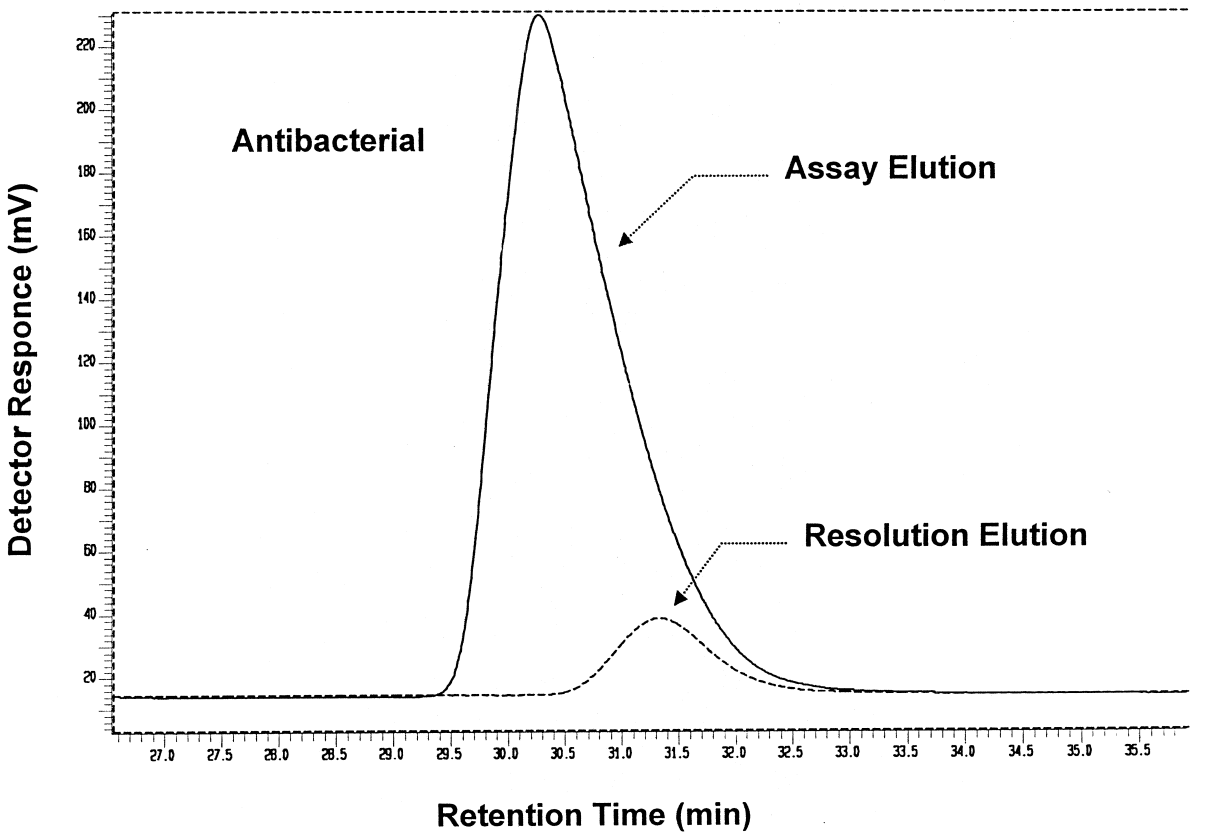

Fig. 3. Elution profiles of the antibacterial – overlay of the resolution injection (dashed line, 3 μg) and the assay injection (solid line, 31.2 μg). Experimental conditions as in Fig. 1.

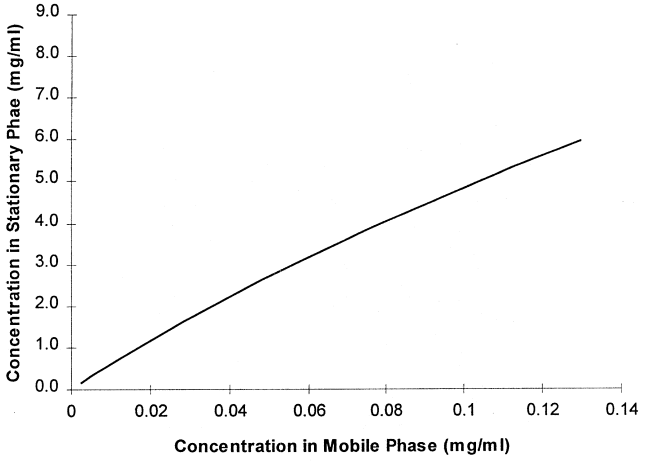

Fig. 4. Isotherm of the antibacterial.

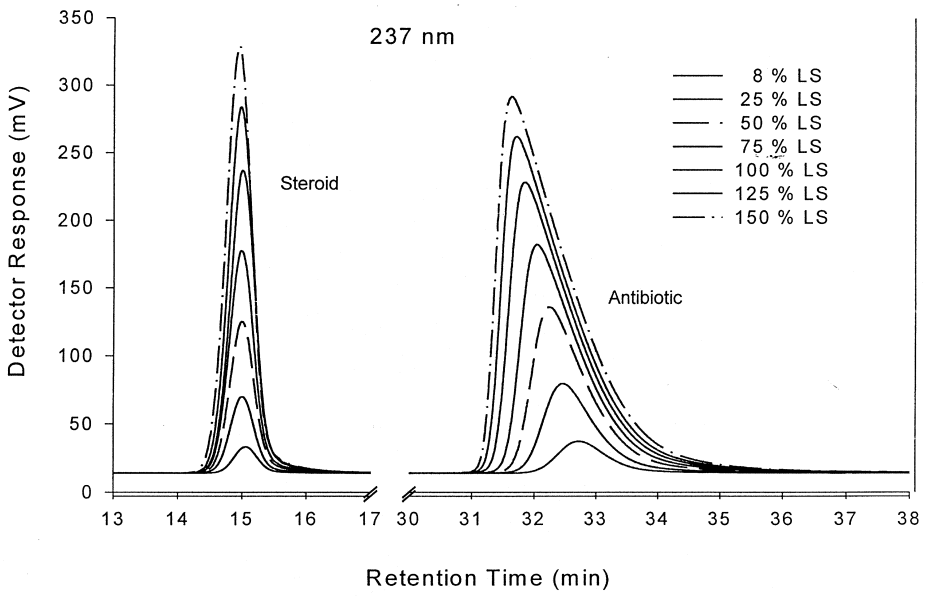

Fig. 5. Elution profiles of standard solutions containing both the steroid and the antibacterial. Experimental conditions as in Fig. 1.

linear range of its isotherm, and we utilized dual-wavelength detection, because not a single wavelength can be found that can simultaneously meet the required S/N ratio for both actives at the 0.05% level (see Fig. 7).

A wavelength of 247 nm, the maximum absorbance wavelength (see Fig. 8) for the steroid is used for its detection, while a wavelength of 237 nm is used for the detection of the antibacterial. The latter is selected because the absorbance at this wavelength is high enough to meet the antibacterial S/N ratio requirement and low enough that a single LOQ solution instead of two is prepared for both actives, thus making the assay procedure simpler and more efficient.

4.4. Linearity and accuracy based on non-symmetric elution peak

The linearity of the chromatographic response is evaluated by correlating the peak area response versus injection concentration for a series of standard solutions containing both actives. The accuracy of the HPLC assay method is evaluated through a recovery study where standard solutions containing formulation excipients were similarly prepared. The linearity and the recovery solutions which were prepared in duplicate, range from 25% to 150% of label strength. The results presented in Fig. 9 for the linearity study and in Fig. 10 for the recovery study demonstrate that the correlation between peak area response and injection concentration is linear with insignificant y-intercept for both actives, although one active is eluted as a symmetric peak and the other as a non-symmetric peak. Acceptable recoveries from formulation excipients are obtained

Table 2
Recovery of drug compound

Concentration, Label strength (%)	Recovery (%)[a]	
	Steroid, 247 nm	Antibacterial, 237 nm
25[b]		100
50	101	101
75	100	99.6
100	100	99.3
125	101	98.4
150	100	97.6

[a] Average of two sets of data.
[b] Not included for the steroid.

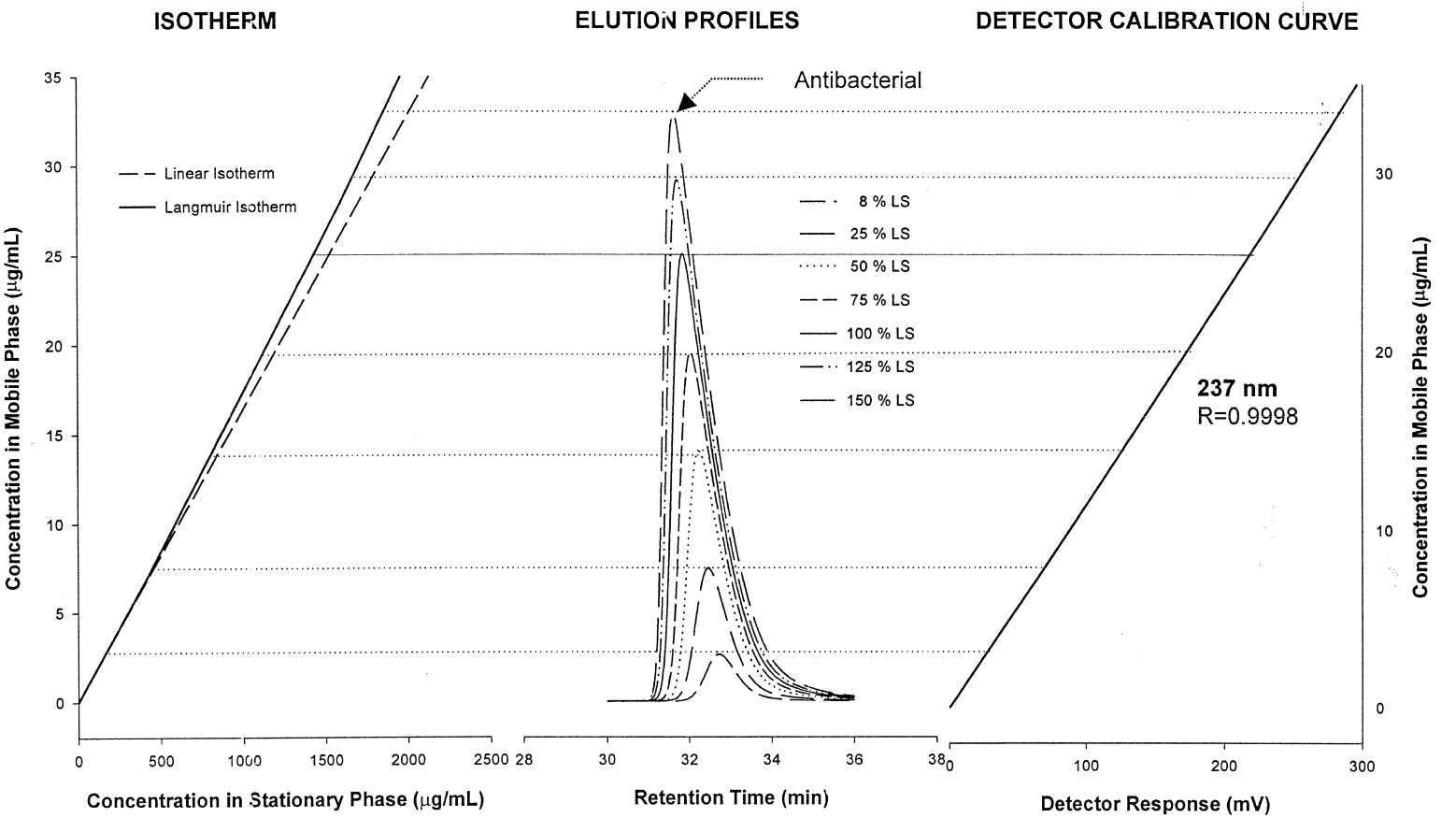

Fig. 6. Relationships of the antibacterial elution concentration to its isotherm and to UV response. Langmuir isotherm: $Q = 61C^{a}(1 + 2.52C)$. Linear isotherm: $Q = 61C$. Detector calibration curve: $C = 0.111401DR^{b} + 0.000023DR$, within the concentration range of 0–35 μg/ml, the linear correlation coefficient for the elution concentration and the detector response is 0.9998. *a*: Elution concentration of the antibacterial (μg/ml), *b*: detector response at 237 nm for the antibacterial (mV).

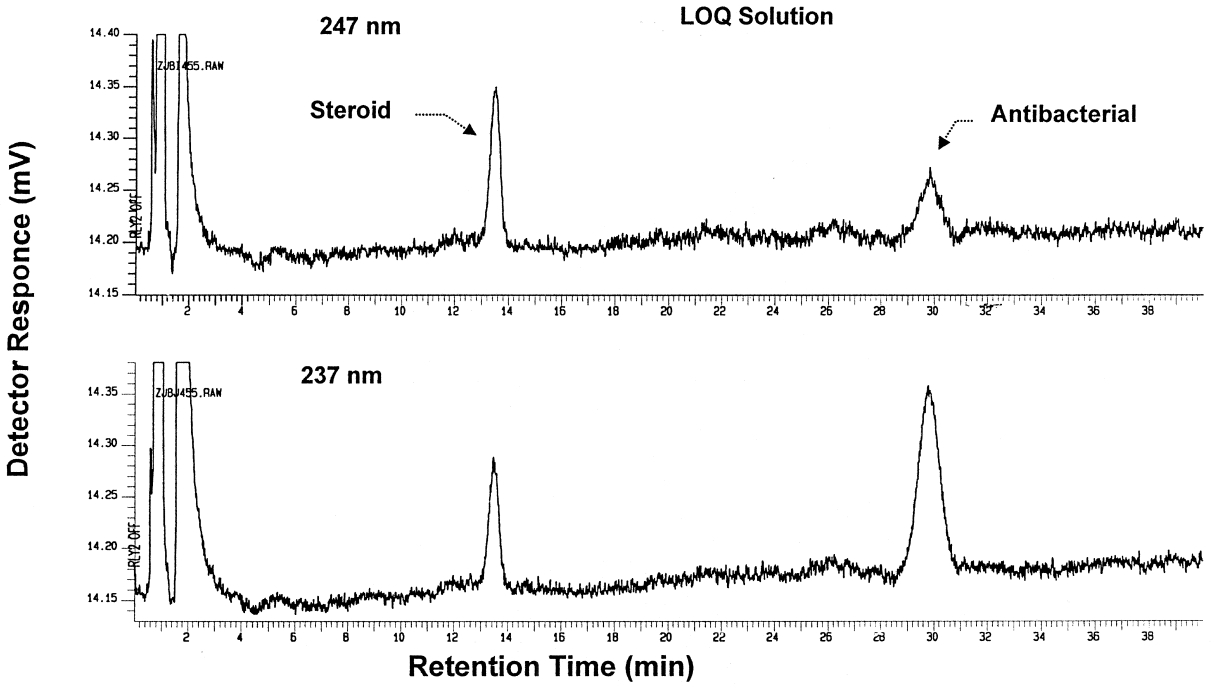

Fig. 7. Elution profile of the LOQ solution, monitored at two different wavelengths (237 nm and 247 nm). Experimental conditions as in Fig. 1.

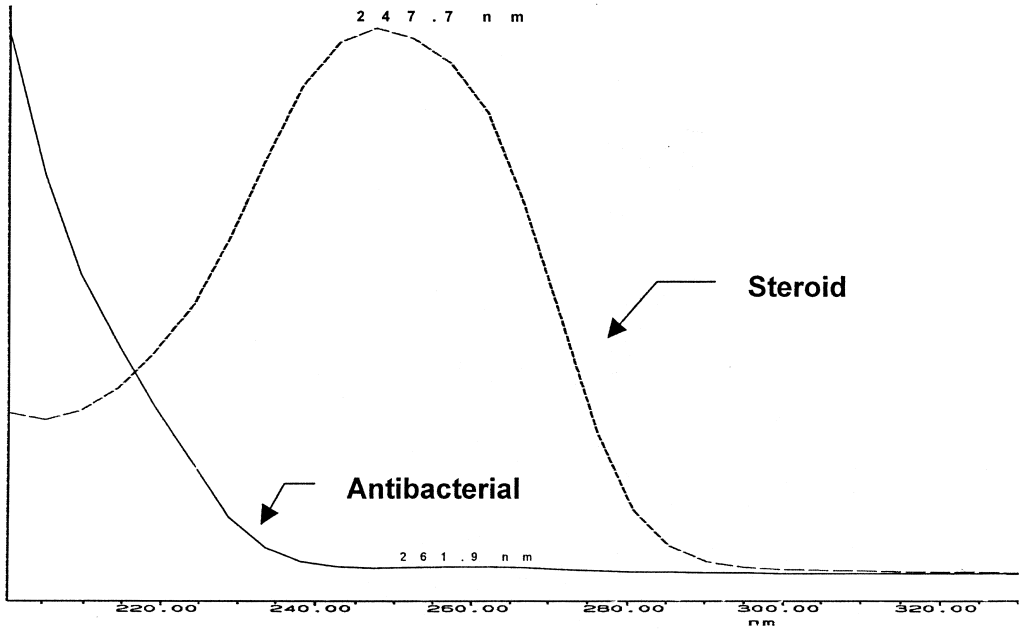

Fig. 8. Ultraviolet absorption spectra of the steroid (dashed line) and the antibacterial (solid line).

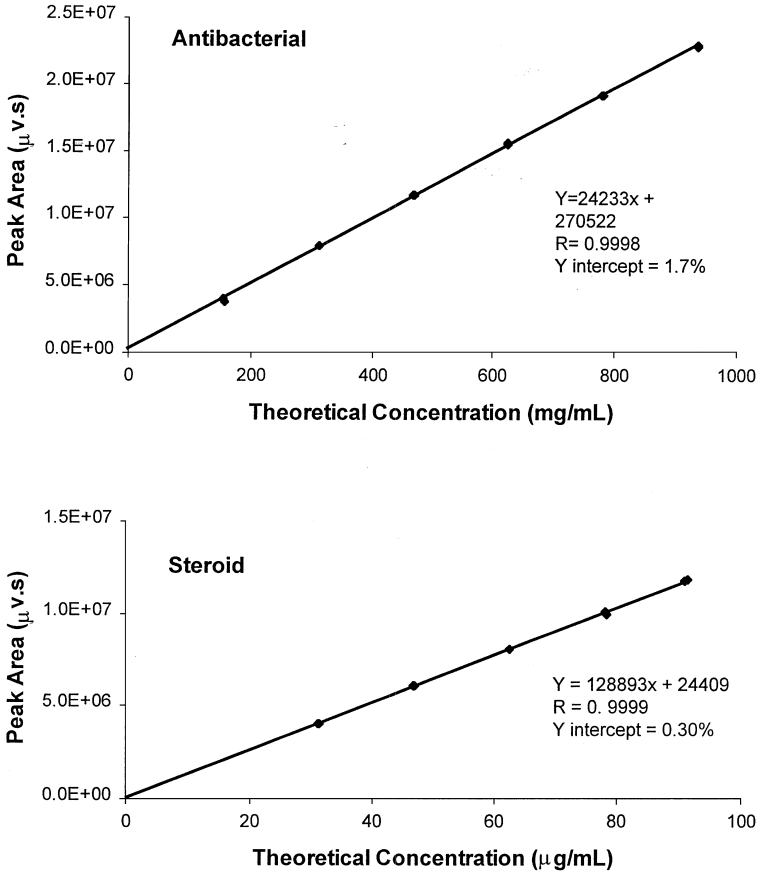

Fig. 9. Linear regression plot for standard solutions containing both the steroid and the antibacterial. Concentration of standard solution: 25%[a], 50%, 75%, 100%, 125% and 150% LS for both drug actives. a: Not included for the steroid.

through the entire range of concentration studied (see Table 2). The results indicate that column overloading does not affect the linearity and the accuracy of the HPLC assay method as long as the elution concentrations are within the linear dynamic range of the UV detector.

Table 3
HPLC system precision

Replicate injection[a]	Peak area (μv s)	
	Steroid	Antibacterial
1	8 227 957	15 976 465
2	8 232 527	15 992 444
3	8 251 877	16 017 594
4	8 267 275	16 050 176
5	8 266 136	16 041 980
Average	8 249 154.4	16 015 731.8
RSD (%)	0.22	0.20

[a] Standard solution at 100% label strength.

4.5. Precision based on non-symmetric elution peak

The precision of the HPLC assay method is demonstrated by the relatively low RSDs obtained for peak area responses of five replicate standard solution injections containing both actives at 100% label strength (Table 3). This is further demonstrated by the same relatively low RSDs which were obtained on eight separate assays of various stability samples (see Table 4 and Fig. 11). The results demonstrate a good HPLC system precision for both

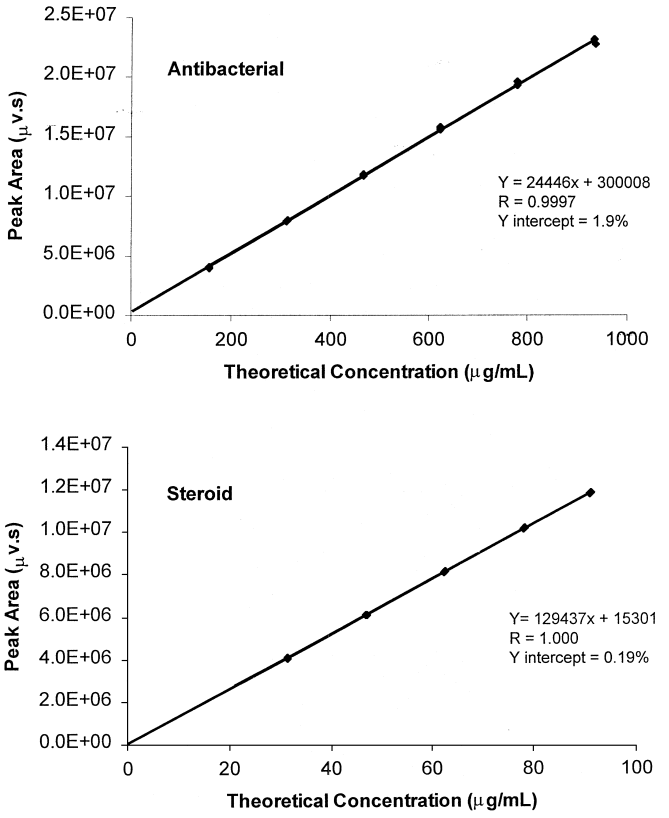

Fig. 10. Linear regression plot for standard plus formulation excipients solutions. Concentrations of sample solutions: 25%[a], 50%, 75%, 100%, 125% and 150% LS for both drug actives. a: Not included for the steroid.

Table 4
Summary of HPLC system precision

HPLC assay, Assay No.[a]	RSD (%) of peak area	
	Steroid	Antibacterial
1	0.22	0.20
2	0.12	0.07
3	0.14	0.15
4	0.27	0.07
5	0.12	0.09
6	0.17	0.18
7	0.51	0.58
8	0.17	0.35

[a] Five replicate injections of a standard solution at 100% label strength.

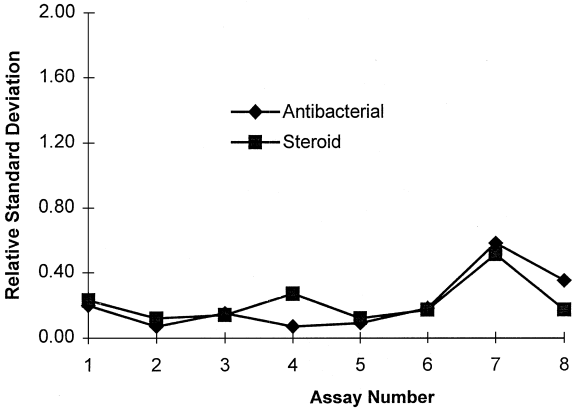

Fig. 11. Relative standard deviations (RSD, %) obtained from five replicate injections of a standard solution containing both drug actives. Experimental conditions as in Fig. 1.

drug actives and show that column overloading does not affect the precision of the HPLC assay method.

4.6. Column performance evaluation for overloaded chromatographic assay

The system suitability tests developed for the overloaded chromatographic system in the subject multi-active formulation are based on a symmetric elution peak, obtained by reducing the injection concentration of the antibacterial at ten times that of the assay concentration. The tailing factor and the resolution factor are calculated from the symmetric peak of the antibacterial because an overloaded asymmetric peak does not correctly reflect column performance. The resolution factor is set high enough to adequately ensure the separation of an early eluting compound from the sharp front of the overloaded antibacterial peak (see Fig. 12).

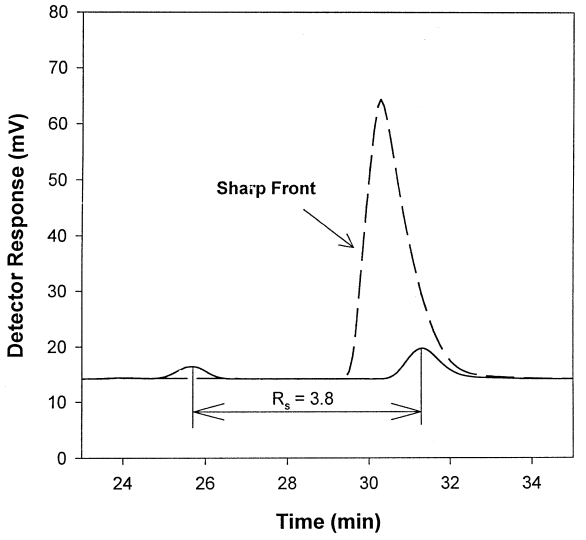

Fig. 12. Demonstration of resolution involving an overloaded elution peak. Solid line: linear elution; dashed line: overloaded elution. Experimental conditions as in Fig. 1.

5. Conclusion

In multi-active pharmaceutical formulations where the label strengths of the actives are at widely different concentrations, a single HPLC method utilizing column overloading can be developed that can assay both actives simultaneously and still achieve a 0.05% LOQ for each active. The non-symmetric peak, whose elution concentration is in the non-linear range of its thermodynamic isotherm, can be assayed as accurately and as precisely as a symmetric Gaussian peak, as long as the elution concentrations are in the linear dynamic range of the UV detector. Although the analytical column is overloaded and a non-symmetric peak is obtained, the HPLC assay can still exhibit good linearity, recovery and reproducibility.

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