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Validated HPLC method for determination of sennosides A and B in senna tablets

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Abstract

This study developed an efficient and reliable ion-pair liquid chromatographic method for quantitation of sennosides A and B in commercial senna tablets. Separation was conducted on a Hypersil C 18 column (250 × 4.6 mm, 5 μm) at a temperature of 40 °C, using a mixture of 0.1 M acetate buffer (pH 6.0) and acetonitrile (70:30, v/v) containing 5 mM tetrahexylammonium bromide as mobile phase. Sennosides A and B were completely separated from other constituents within 14 min. The developed method was validated. Both run-to-run repeatability ($n = 10$) and day-to-day reproducibility ($n = 3$) of peak area were below 0.4% RSD. Linearity of peak area was tested in the range 30–70 μg/ml ($r > 0.9997$). Accuracy was assessed with recovery and the recoveries for sennosides A and B were 101.73 ± 1.30% and 101.81 ± 2.18% ($n = 3 \times 6$), respectively. Robustness of the analytical method was tested using a three-leveled Plackett–Burman design in which 11 factors were assessed with 23 experiments. Eight factors (column, concentration of ion pair reagent, % of organic modifier (acetonitrile), buffer pH, column temperature, flow rate, time constant and detection wavelength) were investigated in a specified range above and below the nominal method conditions. It was found that: (1) column and % acetonitrile affected significantly resolution and retention time, (2) column, % acetonitrile, column temperature, flow rate and time constant affected significantly the plate number of sennoside A, and (3) column and time constant affected significantly the tailing factor.

Keywords: Sennosides A and B determination; Ion-pair HPLC; Validation; Robustness test

1. Introduction

Senna is a crude drug used as laxative and cathartic for the treatment of constipation and for bowel evacuation [1]. It appears in many pharmacopoeias, e.g. British Pharmacopoeia [2], European Pharmacopoeia [3], United States Pharmacopoeia [4] and Japanese Pharmacopoeia [5]. The active constituents of senna are dianthrone glycosides (sennosides A, B, C, D, etc.), free anthraquinones (aloe-emodin, chrysophanol, rhein) and anthraquinone glycosides. Among these constituents, sennosides A and B (Fig. 1) are present in the greatest concentration [6].

The USP uses a fluorometric method to assay the active components of senna preparations. The

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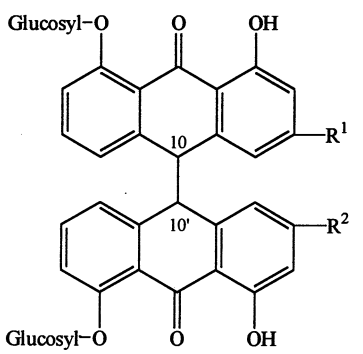
British and European Pharmacopoeias do the same work by means of spectrophotometry. Both fluorometric and spectrophotometric methods are applicable when the total amount of dianthrone and anthraquinone glycosides (free forms also included) is to be counted. However, because the effects of senna leaves are due primarily to sennosides A and B [7,8], it is important to monitor the content of these two components in senna preparations. To accomplish this, some specific method like high-performance liquid chromatography (HPLC) was required. Japanese Pharmacopoeia (JP) uses HPLC to undertake the assay of sennosides A and B in senna leaves [5]. Our laboratory followed the JP procedure but found sennoside A was retained at column for longer than 50 min. This is not suited for a routine analysis.

Many other HPLC methods have been proposed for the specific evaluation of sennosides A and B [9–13]. However, few of them could lead to a simple, rapid and accurate quantitation suitable for their assay in senna preparations. An ion-pair HPLC method was thus developed and then validated for the quantitative assay of sennosides A and B in commercial tablets made of senna extract.



	R^1	R^2	10-10'
Sennoside A	COOH	COOH	*trans*
Sennoside B	COOH	COOH	*meso*

Fig. 1. Structures of sennosides A and B.

2. Experimental

2.1. Apparatus

An HPLC system consisted of a Shimadzu LC-10 AT pump (Kyoto, Japan), a Rheodyne 7725i 20-μl manual injector (Cotati, CA, USA) and a Shimadzu SPD-M10A diode array detector was used. The chromatograms were recorded with a Shimadzu Class-LC 10 HPLC data system on a Pentium II 400 PC compatible computer. Method development was carried out on a Hypersil C-18 column, 5 μm, 250 × 4.6 mm (Phenomenex, Torrance, CA, USA). In robustness test of the developed method, a Luna C-18 column, 5 μm, 250 × 4.6 mm (Phenomenex, Torrance, CA, USA) and a Symmetry C-18 column, 5 μm, 250 × 4.6 mm (Waters, Milford, MA, USA) were also used. A Mettler delta 320 pH meter with an InLab 410 combination electrode (Essex, England) was employed for pH measurement.

2.2. Chemicals and reagents

Sennosides A and B standards were provided by Leiras Oy (Helsinki, Finland). Tetrapentylammonium bromide (tetraamylammonium bromide, TAA) and tetrahexylammonium bromide (THxA) were purchased from Aldrich (Milwaukee, WI, USA). Tetraheptylammonium bromide (THpA) was purchased from Sigma (St. Louis, MO, USA). Acetic acid and sodium acetate were purchased from Merck (Darmstadt, Germany). Sodium hydroxide was purchased from Fluka (Buchs, Switzerland). Sodium bicarbonate was purchased from Riedel-de Haën (Seelze, Germany). Acetonitrile of chromatographic grade was purchased from Baker (Phillipsburg, NJ, USA). Deionized water was obtained from a Barnstead Water Purification System (Dubuque, IA, USA).

2.3. Standard solutions

Sennosides A and B stock solutions were pre-

Table 1
Factors and values of the levels for the P–B design of robustness test

Factors	Lower level (−1)	Nominal level (0)	Upper level (1)
Column	Luna	Hypersil	Symmetry
IPR[a] conc (mM)	4	5	6
ACN (% v/v)	28	30	32
pH of buffer	5.8	6.0	6.2
Column temperature	25°C	40°C	55°C
Flow rate (ml/min)	0.9	1.0	1.1
Time constant (s)	0.32	0.64	1.28
Det. wavelength (nm)	265	270	275
Dummy 1	−1	0	1
Dummy 2	−1	0	1
Dummy 3	−1	0	1

[a] IPR: ion-pair reagent.

pared by dissolving 1.5 mg of the respective compounds in 5 ml of a sodium bicarbonate solution (1 g in 1000 ml of water). After filtration through 0.45 μm Nylon membranes (Whatman), the solutions were diluted to provide standard solutions of sennosides A and B (30, 40, 50, 60 and 70 μg/ml), which were used to make calibration curves.

2.4. Working solution used for method development

Ten tablets of a senna preparation from a pharmaceutical company were ground to fine powders. Five milligrams of the powder were dissolved in 5 ml of sodium bicarbonate solution (1 g in 1000 ml of water). The mixture was sonicated to make sennosides A and B completely dissolve. After filtration through a 0.45 μm Nylon membrane (Whatman), the solution was used as working solution in method development.

2.5. Sample solutions used in assay

The sample solution used to assay sennoside B in tablets was the working solution used in method development. Because the content of sennoside A was less than sennoside B in tablets, 15 mg of powder was taken to prepare the sample solution used to assay sennoside A in tablets, other procedures being the same as in Section 2.4.

Tablets used for method development and validation were obtained from a pharmaceutical company.

2.6. Chromatographic conditions

An acetate buffer of pH 6.0 was prepared by adding 1 M sodium acetate to 1 M acetic acid. The solution after diluting 10 times was mixed with acetonitrile (70/30, v/v). An accurately weighed tetrahexylammonium bromide (THxA) of 2.17 g was dissolved in 1000 ml of the above mixture and the resulting solution was used as the mobile phase of HPLC. Flow rate of 1.0 ml/min, injection volume of 20 μl, detection wavelength at 270 nm, and column temperature at 40 °C were used in the method.

2.7. Robustness test of method

A Plackett–Burman (P–B) design was employed to test the robustness of the method. Eight factors, i.e. column, concentration of ion-pair reagent, volume percentage of organic modifier, pH of buffer solution, column temperature, mobile phase flow-rate, detector time constant, and detection wavelength, were tested. Each factor took three levels (Table 1). Values of the levels were set according to our experience and literature suggestions [14–16]. Those of nominal levels were the optimum conditions developed in this study.

A P–B design with N experiments can examine up to $N-1$ factors, where N is a multiple of 4. In this test N is 12 and three factors $(11-8)$ were assigned to dummy factors. Because each factor had three levels, the P–B design was divided into two fractions: Matrix 1 and Matrix 2. Matrix 1 comprised the 12 experiments where the factors

were changed from nominal to lower levels. Matrix 2 comprised another 12 experiments where factors were changed from nominal to upper levels. Matrix 1 expressed with the coded factors (0 as nominal levels and -1 as lower levels) is listed in Table 2 [17]. Matrix 2 was just a reflection of Matrix 1, with code -1 in Matrix 1 being changed to 1 in Matrix 2.

Results of the experiments were evaluated with four responses. They were (1) resolution between the critical pair (sennoside B and compound X), (2) retention time of sennoside A, (3) peak efficiencies of sennosides A and B, and (4) tailing factors of sennosides A and B peaks.

Resolution (R_s) was calculated by the equation:

$$R_s = 2(t_2 - t_1)/(w_1 + w_2)$$

where t_1 and t_2 are retention times, w_1 and w_2 are peak widths of sennoside B and compound X, respectively. Retention time of sennoside A was taken as the analysis time because it was the latest-eluting compound. Peak efficiencies were obtained by calculating plate numbers (N) with the equation:

$$N = 5.54(t/w_{0.5})^2$$

where t is retention time and $w_{0.5}$ is the peak width at its half height.

Tailing factors (T) were calculated by the equation:

$$T = w_{0.05}/2f$$

where $w_{0.05}$ is the peak width at its 5% height from the baseline and f is the distance from the peak maximum to the leading edge of the peak at 5% peak height.

The effect of each factor on response was calculated as:

$$E_{x(0,-1)} = \frac{\sum Y(-1)}{N/2} - \frac{\sum Y(0)}{N/2}$$

$$E_{x(0,+1)} = \frac{\sum Y(+1)}{N/2} - \frac{\sum Y(0)}{N/2}$$

where $E_{x(0,-1)}$ and $E_{x(0,+1)}$ are the effects of factor X on response Y when factor X is changed from level 0 to -1 and 0 to $+1$, respectively. $\Sigma Y(-1)$, $\Sigma Y(0)$ and $\Sigma Y(+1)$ are the sums of the responses where factor X is at levels -1, 0 and $+1$, respectively. N is the number of experiments in the design and equal to 12.

The standard error of the experiments is calculated as:

Table 2
Matrix 1 of the Plackett–Burman design for 11 factors ($N = 12$ experiments)

Experiment	Factors										
	A	B	C	D	E	F	G	H	I	J	K
1	−1	−1	0	−1	−1	−1	0	0	0	−1	0
2	0	−1	−1	0	−1	−1	−1	0	0	0	−1
3	−1	0	−1	−1	0	−1	−1	−1	0	0	0
4	0	−1	0	−1	−1	0	−1	−1	−1	0	0
5	0	0	−1	0	−1	−1	0	−1	−1	−1	0
6	0	0	0	−1	0	−1	−1	0	−1	−1	−1
7	−1	0	0	0	−1	0	−1	−1	0	−1	−1
8	−1	−1	0	0	0	−1	0	−1	−1	0	−1
9	−1	−1	−1	0	0	0	−1	0	−1	−1	0
10	0	−1	−1	−1	0	0	0	−1	0	−1	−1
11	−1	0	−1	−1	−1	0	0	0	−1	0	−1
12	0	0	0	0	0	0	0	0	0	0	0

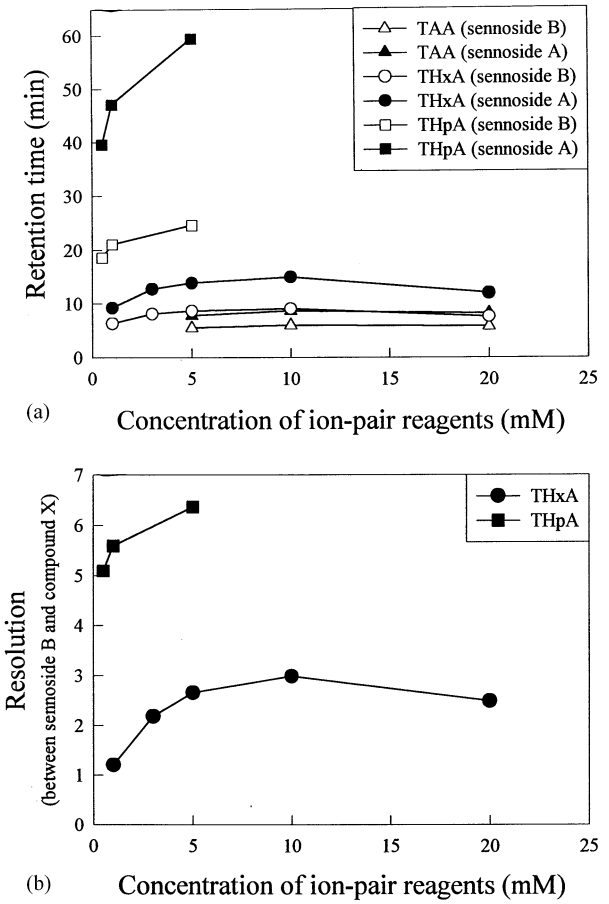


Fig. 2. (a) Effect of different ion-pair reagents on retention time of sennosides A (solid symbols) and B (empty symbols). (b) Effect of different ion-pair reagents on resolution between sennoside B and compound X (for TAA the resolutions were too small to be traced). Column: Hypersil C-18 (250 × 4.6 mm, 5 μm). Mobile phase: 0.1 M acetate buffer (pH 6.0)/ACN (70:30, v/v), various concentrations of ion-pair reagents. Flow rate: 1 ml/min. Temperature: ambient. Detection: 270 nm. TAA, tetraamylammonium bromide; THxA, tetrahexylammonium bromide; THpA, tetraheptylammonium bromide.

$$\text{S.E.} = \sqrt{\frac{\sum (E_{\text{D}i})^2}{n_i}}$$

where n_i and $E_{\text{D}i}$ are number and effect of dummy factors, respectively. The factor is considered to have significant effect on a response at 5% level if $t_{\text{calc}} > t_{\text{critical}(\alpha = 0.05)}$, where $t_{\text{calc}} = |E_x|/(\text{SE})$. If $t_{\text{calc}} > t_{\text{critical (a = 0.01)}}$ the effect of the factor is significant at 1% level.

3. Results and discussion

3.1. Method development

All the previous HPLC methods [9–13] were undertaken with reversed-phase chromatography, two of which [9,12] used the ion-pair mode. JP also employed the ion-pair chromatography (IPC) to carry out the assay [5]. The merits of IPC are as follows. First, it is an isocratic system that is more simple and stable than a gradient system and thus more suitable for QC and routine analysis. Secondly, senna extracts are a complex mixture and it is difficult to get a good separation among the relevant compounds. By simultaneous varying concentration of the ion-pair reagents and pH, considerable control is achievable over both retention range and band spacing [18]. Reverse-phase IPC was therefore implemented in this study.

Preliminary experiments on sample solution showed the resolution between sennoside B and its neighboring unknown compound (for convenience' sake, named as compound X) was critical to the over-all separation. In the text of this paper whenever the resolution is mentioned, unless specified in other way, it always refers to this critical pair.

3.1.1. Effect of ion-pair reagent species and concentration

Based on JP procedure, tetraalkylammonium salts were used as ion-pair reagents in this study. Because ion-pair reagents of different lengths would cause different extent of retention, three homologous tetraalkylammonium salts, i.e. tetrapentylammonium bromide (TAA), tetrahexylammonium bromide (THxA) and tetraheptylammonium bromide (THpA), were tested for their suitability as the ion-pair reagent for separation. The results (Fig. 2a) showed that for THpA the retention time of sennoside A lasted too long. For TAA the resolution (of course, between sennoside B and compound X) was too small (not shown). For THxA retention time (Fig. 2a) and resolution (Fig. 2b) were both acceptable.

In Fig. 2b, at 10 mM of THxA the resolution was the greatest (3.0), however, the retention time (of sennoside A) was also the longest (14.9 min). The concentration of 5 mM was a good compromise.

3.1.2. Effect of percentage of organic solvent

With reference to JP, acetonitrile was used as the organic modifier making up the mobile phase. The effect of acetonitrile on separation was studied with different amount of composition (26–34%, v/v). Results showed that when acetonitrile content was lower than 30%, retention times increased rapidly (Fig. 3a), and when acetonitrile content was higher than 30%, resolution dropped below the level of a base-line separation (Fig. 3b). 30% of acetonitrile was therefore a suitable amount added in mobile phase.

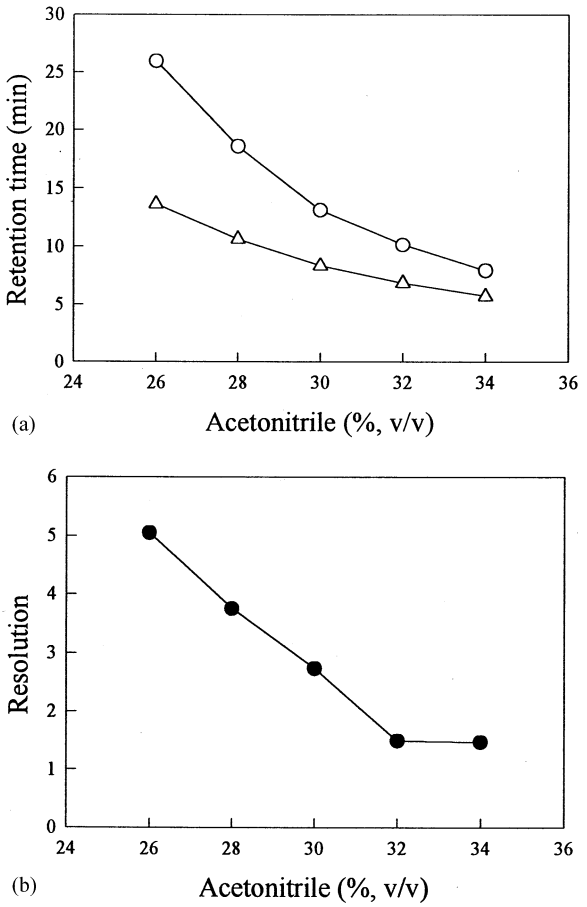


Fig. 3. (a) Effect of acetonitrile percentage on retention time of sennoside A (○) and sennoside B (△). (b) Effect of acetonitrile percentage on resolution between sennoside B and compound X. Mobile phase: 0.1 M acetate buffer (pH 6.0)/various volume% of ACN, 5 mM THxA. Other conditions are as described in Fig. 2.

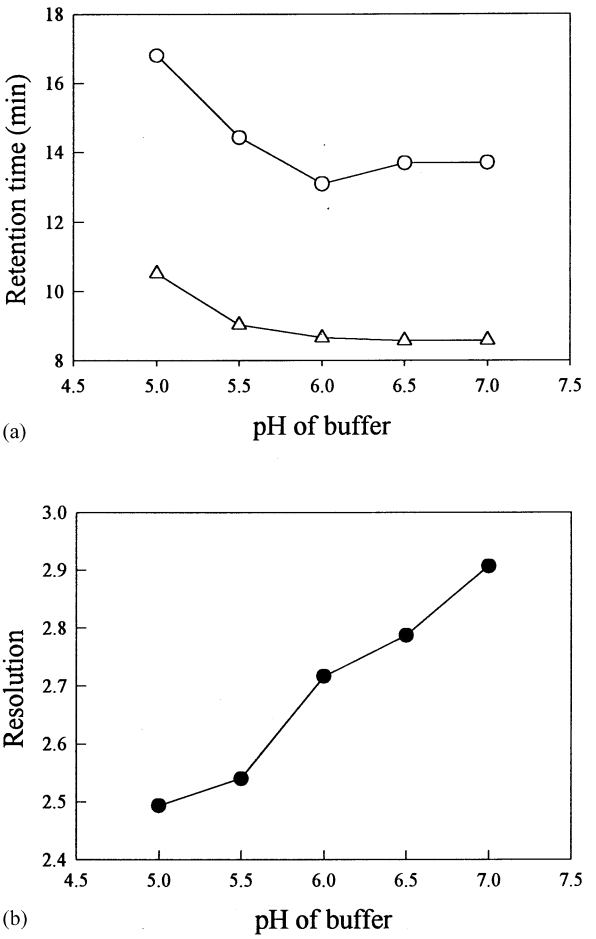


Fig. 4. (a) Effect of buffer pH on retention time of sennoside A (○) and sennoside B (△). (b) Effect of buffer pH on resolution between sennoside B and compound X. Mobile phase: 0.1 M acetate buffer (pH 5.0–7.0)/ACN (70:30, v/v), 5 mM THxA. Other conditions are as described in Fig. 2.

3.1.3. Effect of buffer pH

Buffer pH affected the ionization of the carboxyl groups of sennosides A and B. This in turn affected the ion-pair formation and retention of the solutes. A pH range of 5.0–7.0 was examined

and the results are shown in Fig. 4. Although the resolution increased steadily over the tested range (Fig. 4b), the retention time of sennoside A reached a valley at pH 6.0 (Fig. 4a). Besides, a minute peak entered into that of sennoside B at pH 6.5. Therefore 6.0 was regarded as the optimum of buffer pH.

Other minor factors including temperature, wavelength and flow rate were all investigated and their optimum values had been found. Chromatogram of sennoside A and B standards and their on-line UV spectra are shown in Fig. 5.

3.2. Method validation

3.2.1. Specificity

The chromatogram obtained from the sample solution is shown in Fig. 6. The last-eluting compound was sennoside A (t_R about 13 min), which was largely separated from its neighboring peak. Resolution between sennoside B and compound X (peak 8) attained 2.7, which was beyond the requirement of a base-line separation. UV spectra of upslope, apex and downslope positions of

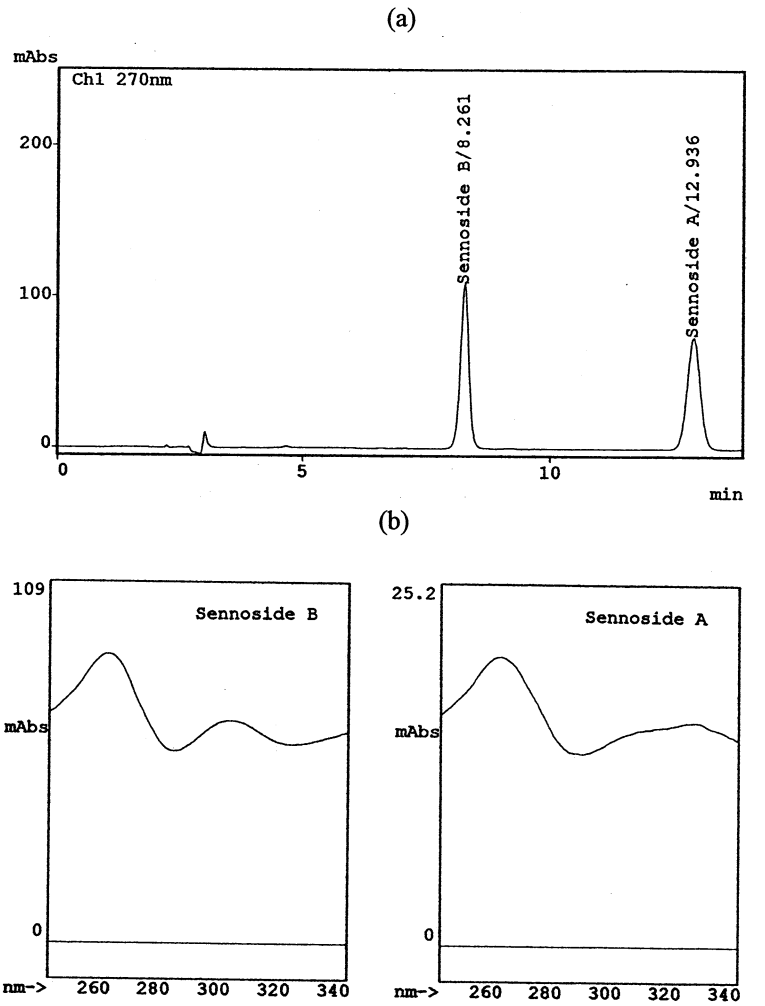


Fig. 5. (a) Chromatogram obtained from sennosides A and B standards. (b) On-line UV spectra of sennosides A and B standards. Hypersil C-18 (250 × 4.6 mm, 5 μm). Mobile phase: 0.1 M acetate buffer (pH 6.0)/ACN (70:30, v/v), 5 mM THxA. Flow rate: 1 ml/min. Temperature: 40 °C. Detection: 270 nm.

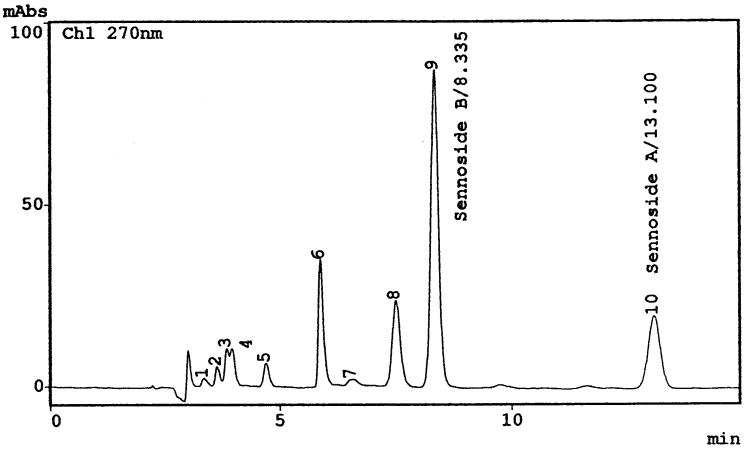


Fig. 6. Chromatogram obtained from senna tablet extractions. Conditions are as described in Fig. 5. Peak 8 is of compound X.

peaks 9 and 10 were well overlaid with each other and with those of the sennosides A and B standards.

In Fig. 7 were listed the spectra of all the peaks appearing in the chromatogram of Fig. 6, exempting the very minor peaks. It could be seen the spectra of some peaks look much like those of sennosides A and B (peaks 9 and 10), for examples, peaks 3,4,5 and 8. We supposed these were also due to dianthrone or anthraquinone derivatives.

3.2.2. Precision

Run-to-run repeatability and day-to-day reproducibility were used to assess the precision of repeated injections. Ten injections were made each day and this was repeated for 3 consecutive days. Relative standard deviations (RSD) of retention time of sennoside A were 0.03 and 0.23% for repeatability and reproducibility, respectively. Those of sennoside B were 0.03 and 0.09%. RSD of peak area of sennoside A were 0.32 and 0.37% for repeatability and reproducibility, respectively. Those of sennoside B were 0.24 and 0.28%. The tested concentration was 30 μg/ml for both sennosides A and B.

The precision of the method was assessed in the accuracy test stated below.

3.2.3. Linearity

Five standard solutions (30, 40, 50, 60 and 70 μg/ml) were run for the calibration curve. In each day of 3 consecutive days the calibration curves were made. Their coefficients of correlation (r) were all above 0.9997. After passing the statistical test that proved no difference between them [19], the three lines were combined to form a new line for quantitative use. The equations were $y = 27684.08x - 9277.37$ and $y = 25918.21x + 26525.93$ for sennosides A and B, respectively (y for peak area and x for concentration in μg/ml).

3.2.4. Accuracy

The accuracy of the method was evaluated with recovery test. The sample solutions were prepared to contain sennosides A and B in about 45 μg/ml, respectively. Equal volumes of solutions containing sennosides A and B in 30 μg/ml were then added. The recoveries were between 98.95 and 104.94%.

To test the repeatability and reproducibility of the recoveries, the above procedures were repeated six times a day for 3 consecutive days. RSD of recovery of sennoside A were 0.99 and 1.28% for repeatability and reproducibility, respectively. Those of sennoside B were 1.39 and 2.14%.

3.2.5. Stability of solutions

The stability of solutions was tested with standard solutions and sample solutions that were stored at 4 °C and at room temperature (about 25 °C) for 24 h. No significant changes in concentrations of sennosides A and B were observed.

3.2.6. Robustness

The results of the Plackett–Burman design are listed in Tables 3 and 4. The effects of each factor were calculated. The calculated t-values of the effects of factors on responses are listed in Tables 5 and 6. For easy understanding the bar diagrams

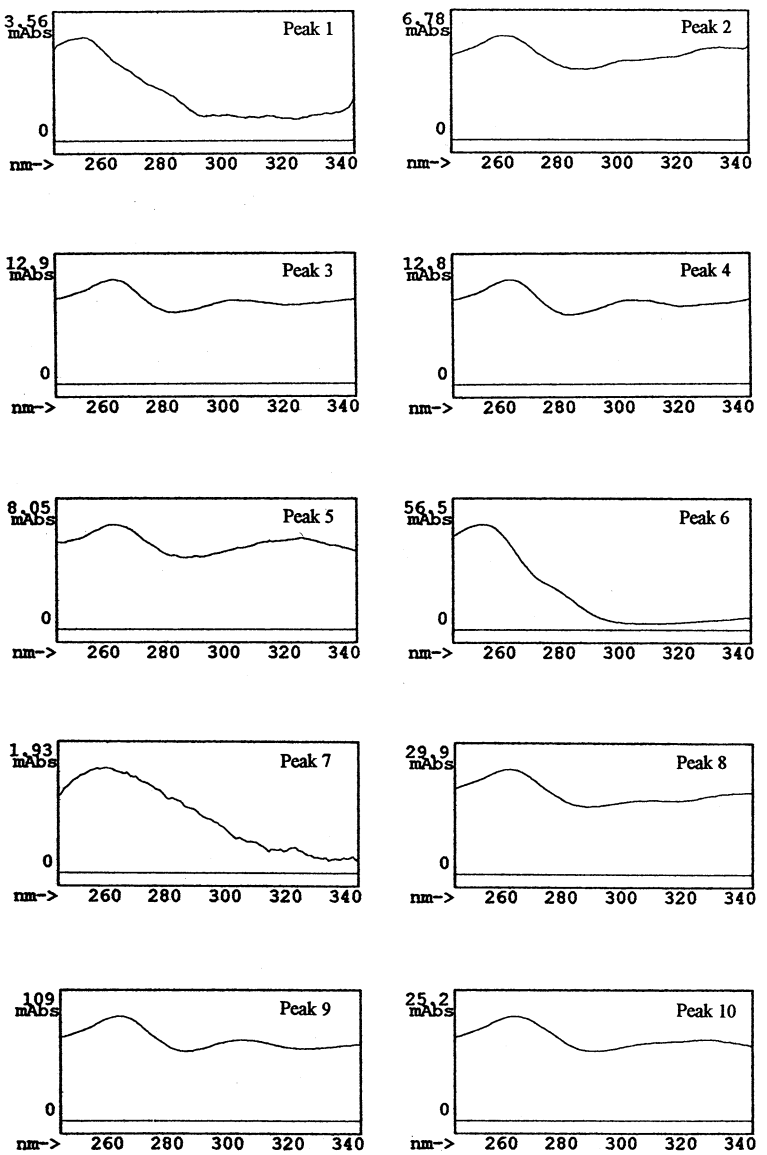


Fig. 7. On-line UV spectra of the peaks appearing in the chromatogram of Fig. 6.

Table 3
Experimental results of the Matrix 1 of the P–B design

Experiment	Response					
	R_s	t_R (min)	N (sen. B)	N (sen. A)	T (sen. B)	T (sen. A)
1	3.41	25.61	11 043	16 208	1.04	1.06
2	3.88	20.57	11 836	12 593	1.01	1.13
3	5.41	36.71	15 893	17 874	0.95	1.03
4	2.62	14.16	8031	11 258	1.01	1.13
5	3.80	20.90	10 651	12 179	1.07	1.12
6	3.04	16.98	12 591	12 815	1.02	1.03
7	4.10	22.86	16 738	15 417	0.93	1.07
8	3.29	23.50	11 942	16 054	1.02	1.03
9	5.06	31.12	14 867	16 652	0.94	1.01
10	4.02	20.27	11 047	11 931	1.02	1.05
11	5.06	34.60	13 424	15 939	0.99	1.05
12	2.69	13.01	12 791	11 183	1.04	1.09

Table 4
Experimental results of the Matrix 2 of the P–B design

Experiment	Response					
	R_s	t_R (min)	N (sen. B)	N (sen. A)	T (sen. B)	T (sen. A)
1	1.43	16.75	9667	12 122	1.02	0.98
2	1.55	10.32	7905	8510	1.45	1.21
3	1.37	12.71	6602	9941	1.29	1.17
4	2.27	14.59	8491	9944	1.24	1.13
5	1.54	9.77	6925	10 397	1.13	1.02
6	2.03	12.74	7544	8973	1.35	1.17
7	2.45	18.63	9273	11 021	1.21	1.03
8	3.07	18.52	10 468	12 948	1.04	1.02
9	1.71	14.73	7824	10 860	1.25	1.12
10	1.64	10.91	5882	10 745	1.14	1.06
11	1.57	13.47	8969	11 746	1.07	1.00
12	2.64	12.95	12 045	11 722	0.98	1.06

are illustrated (Figs. 8 and 9). Based on these data, the effects of the factors were discussed.

3.2.6.1. Effect of columns. Resolution was affected by the change of columns, but the effect was unidirectional (Fig. 8a); only when the column was changed from nominal to lower level, was its effect significant. No matter what column was used, retention time and plate number of sennoside A were greatly influenced (Fig. 9a and b). This is in full accordance with our previous observation that the Symmetry and Luna columns retained the sennosides far more than the Hypersil column did. It was probably due to this prolonged retention that caused the plate number of sennoside A to be higher. When column changed, tailing factor was also affected; Luna column rendered the peaks of sennosides more symmetrical while the Symmetry column did not (Fig. 8c and Fig. 9c).

3.2.6.2. Effect of THxA concentration. When concentration of THxA (ion-pair reagent) was decreased from 5 to 6 mM, resolution fell off (at 5% level) (Fig. 8a). However, when concentration in-

creased from 5 to 6 mM, resolution remained unaffected. This could be attributed to the saturation of ion-pair reagent on surface of stationary phase at concentrations above 5 mM. To other responses, concentration of THxA had no effect.

3.2.6.3. Effect of acetonitrile percentage. Change of percentage of acetonitrile in mobile phase brought about great influence on both resolution and retention time. Its lessening (from 30 to 28%, v/v) helped the resolution to the greatest extent (Fig. 8a), however, at the expense of terri-

Table 5
Calculated t values of the effects of factors (t_{calc}) on resolution (R_s), retention time (t_R), plate no. (N) and tailing factor (T) from results of Matrix 1 of the P–B design

Factor	t_{calc}					
	R_s	t_R (min)	N (sen. B)	N (sen. A)	T (sen. B)	T (sen. A)
Column	12.16**	19.95**	3.11	61.03**	−6.56**	−3.35*
IPR conc.	−3.52*	−2.87	−2.44	−1.66	1.22	0.22
ACN%	15.67**	13.99**	0.84	9.86**	−1.67	−0.32
Buffer pH	1.43	4.77*	−1.25	4.54*	0.33	−1.08
Temp. column	−1.26	−0.84	−1.36	−6.80**	1.22	3.46*
Flow rate	−1.43	2.40	−0.54	12.45**	3.89*	0.11
Time constant	3.54*	1.31	1.66	7.26**	−7.44**	0.00
Wavelength	0.17	−1.01	−0.41	−1.58	−1.22	0.43
D1	−1.24	0.65	−1.44	−0.72	1.22	−0.76
D2	0.93	−1.40	0.55	0.70	−0.11	−1.30
D3	0.77	−0.79	0.79	−1.41	−1.22	−0.86
Critical values	$t_{(0.05,3)}$	3.18				
	$t_{(0.01,3)}$	5.84				

* Significant at 5% level.
** Significant at 1% level.

Table 6
Calculated t values of the effects of factors (t_{calc}) on resolution (R_s), retention time (t_R), plate no. (N) and tailing factor (T) from results of Matrix 2 of the P–B design

Factor	t_{calc}					
	R_s	t_R (min)	N (sen. B)	N (sen. A)	T (sen. B)	T (sen. A)
Column	−0.05	11.41**	0.91	9.70**	−2.02	−2.76
IPR conc.	0.06	2.71	−0.26	1.54	0.57	0.54
ACN%	−3.26*	−10.80**	−3.05	−5.26*	2.36	1.61
Buffer pH	−1.91	−1.82	−1.66	−2.31	0.27	0.37
Temp. column	−1.19	0.48	0.20	−1.68	0.37	−1.86
Flow rate	−0.95	−2.17	−0.77	−3.65*	1.92	1.44
Time constant	−0.37	0.66	−1.44	−12.12**	7.09**	5.90**
Wavelength	1.02	2.02	−1.44	1.24	−0.37	−0.87
D1	0.79	0.75	−0.26	0.94	−0.02	−0.37
D2	−1.20	0.47	−1.68	−0.81	0.22	−1.69
D3	0.97	1.49	−0.34	−1.21	1.72	0.04
Critical values	$t_{(0.05,3)}$	3.18				
	$t_{(0.01,3)}$	5.84				

* Significant at 5% level.
** Significant at 1% level.

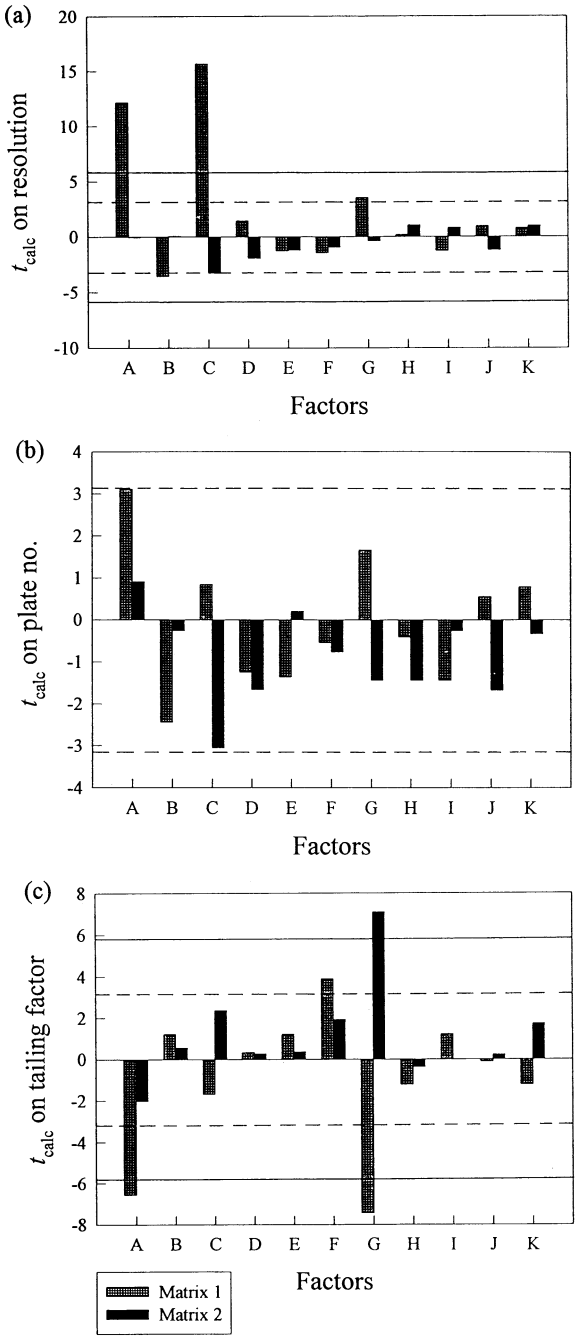


Fig. 8. Bar diagrams showing the calculated t values (t_{calc}) of the effects of factors on (a) resolution (between sennoside B and compound X), (b) plate no. of sennoside B, and (c) tailing factor of sennoside B. A: Column, B: IPR conc., C: ACN%, D: pH of buffer, E: Column temp., F: Flow rate, G: Time constant, H: Det. wavelength, I–K: Dummy 1–3. ---, $t_{critical}$ at 5% level. —, $t_{critical}$ at 1% level.

ble prolongation of retention time (Fig. 9a). Besides, the plate number of sennoside A was affected by the change (Fig. 9b). This might have something to do with the change of retention time.

3.2.6.4. Effect of buffer pH. The upward going of buffer pH (from 6.0 to 6.2) had no effect on each response. However, when pH went downward (from 6.0 to 5.8), the retention time and plate number of sennoside A were somewhat affected (at 5% level) (Fig. 9a and b). Time was prolonged and plate number was increased.

3.2.6.5. Effect of column temperature. Change of column temperature only had influence on the plate number of sennoside A (Fig. 9b), the latest-eluting compound: as the temperature was lowered (from 40 to 25 °C), the plate number of sennoside A decreased. This could be attributed to the loss of efficiency in mass transfer.

3.2.6.6. Effect of mobile phase flow-rate. Peak efficiency of sennoside A was obviously affected by the change of mobile phase flow-rate, especially when the flow-rate was slowed from 1.0 to 0.9 ml/min; plate number was heightened significantly (Fig. 9b). To sennoside B, this slowdown disfavored its peak symmetry (at 5% level) (Fig. 8c).

3.2.6.7. Effect of detector time constant. Five values of time constant can be selected in the detector settings. They are 0.24, 0.32, 0.64, 1.28 and 2.00 s. The medium value 0.64 was used in our routine works and therefore it was taken for the nominal level. From Fig. 8c, it could be seen the peak tailing of sennoside B is greatly affected when the time constant is changed (either moved downward or upward). Decrease of time constant significantly favors the peak symmetry of sennoside B. For sennoside A, it is also noted (Fig. 9c) the peak symmetry went badly when the time constant was increased. As for the lack of influence on peak symmetry of sennoside A when the time constant was shortened (from 0.64 to 0.32 s), it could be attributed to the peak broadening of sennoside A as a result of late-eluting (see Fig. 5). Beside peak asymmetry, the plate number of the late-eluter, sennoside A, was also affected by the change of time constant.

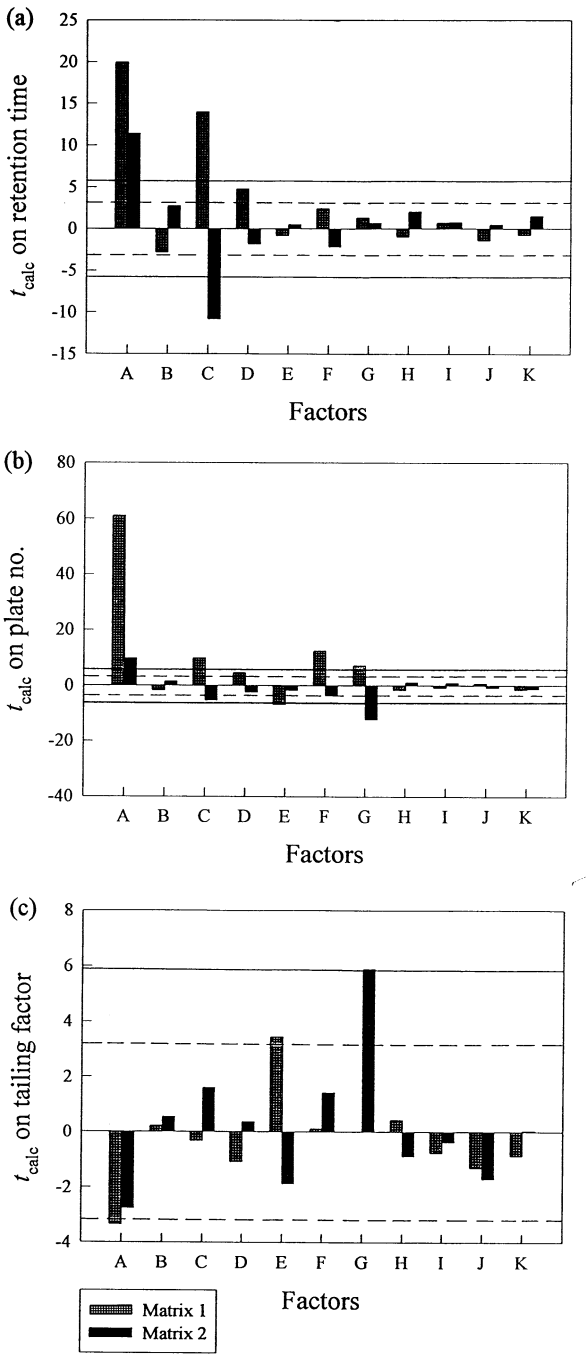


Fig. 9.

3.2.6.8. Effect of detection wavelength. Change of detection wavelength at 5 nm interval above and below the nominal level (270 nm) did not have any effect on each of the responses.

To summarize the above observations, three factors, namely, the column (manufacturer), acetonitrile percentage, and detector time constant were found to affect most prominently the responses within their ranges tested, the other factors being less (column temperature and flow rate) or not effective. More attention should be paid to these factors when the method is to be repeated or transferred. To avoid the bias of the column which is a qualitative (discontinuous) factor in nature, the whole P–B design (23 experiments) was repeated with the exchange of the columns (the lower level being Symmetry column and the upper level being Luna column). The results were almost identical, with only minor differences.

3.3. Assay of the tablets

The contents of sennosides A and B in a tablet (average weight 139.3 ± 5.0 mg) were 1.93 ± 0.01 mg ($n = 3$) and 5.44 ± 0.03 mg ($n = 3$), respectively. The combined weight of sennosides A and B was 7.37 ± 0.02 mg ($n = 3$).

4. Conclusions

Sennosides A and B are the major constituents and the main pharmacologically active principles of senna extracts and its preparations. The analytical method developed in this study is specific for sennosides A and B, which allowed the assay of these two compounds, either in single or in a combined amount.

Because of the strict requirement of extensively validated method by regulatory authorities, robustness testing is now widely applied in the

Fig. 9. Bar diagrams showing the calculated t values (t_{calc}) of the effects of factors on (a) retention time of sennoside A, (b) plate no. of sennoside A, and (c) tailing factor of sennoside A. A: Column, B: IPR conc., C: ACN%, D: pH of buffer, E: Column temp., F: Flow rate, G: Time constant, H: Det. wavelength, I–K: Dummy 1–3. ---, $t_{critical}$ at 5% level. —, $t_{critical}$ at 1% level.

pharmaceutical world. Results of the robustness test showed that the method was susceptible mainly to the change of column brand (manufacturer), acetonitrile percentage in mobile phase, and detector time constant. Care should be taken with these factors whenever the method is applied.

After validation with the various items, especially the robustness test, the developed method should be applicable for the QA/QC assays.

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