JPP Journal of Pharmacy and Pharmacology

JPP 2001, 53: 323–331

Received August 9, 2000
Accepted October 24, 2000
ISSN 0022-3573

Control of impurities in diphenhydramine hydrochloride by an ion-pairing, reverse-phase liquid chromatographic method

L. Henderson, J. H. McB. Miller and G. G. Skellern

Abstract

A precise, sensitive, repeatable and robust reverse-phase liquid chromatographic method has been developed for the control of seven possible impurities of diphenhydramine hydrochloride. The robustness of the method was examined by varying, in turn, each of four mobile-phase parameters (acetonitrile content, buffer salt concentration, ion-pair reagent concentration and pH). The method was linear in the range 0–0.14 mg mL^{-1} for diphenhydramine hydrochloride with an acceptable precision and accuracy, and a limit of detection of 0.17 μg mL^{-1}. Five samples of diphenhydramine hydrochloride from two sources were analysed with the developed liquid chromatographic method.

Introduction

Diphenhydramine hydrochloride, 2-diphenylmethoxy-*N*, *N*-dimethylethylamine hydrochloride (Figure 1, **I**), is an antihistamine commonly found in cough and cold preparations. Potentially there are seven manufacturing or degradation impurities: α-phenylbenzenemethanol (**II**); benzophenone (**III**); 1,1′-methylenebis[benzene] (**IV**); 2-diphenylmethoxy-*N*-methylethylamine (**V**); *N*, *N*-dimethylethyl-2-[(2-methylphenyl) methoxy]ethylamine (**VI**); *N*, *N*, *N*′-trimethyl-*N*′-[2-(diphenylmethoxy) ethyl]ethylenediamine (**VII**) or 2-[(4-bromophenyl)phenylmethoxy]-*N*, *N*-dimethylethylamine (**VIII**).

In this study we compared a liquid chromatographic method developed by the authors with an existing thin-layer chromatographic method (*European Pharmacopoeia* 1997) for the control of potential impurities of diphenhydramine hydrochloride. Our liquid chromatographic method, which was fully validated, was also compared with another liquid chromatographic method, similar to one currently described in the *United States Pharmacopeia* (1995) monograph for diphenhydramine hydrochloride, for its ability to completely resolve diphenhydramine hydrochloride from its seven potential impurities. The preferred method was employed to compare the impurity profiles of diphenhydramine hydrochloride from different manufacturers.

Division III EDQM, Council of Europe, F-67029 Strasbourg Cedex 1, France

L. Henderson, J. H. McB. Miller

Department of Pharmaceutical Sciences, SIBS, University of Strathclyde, 27 Taylor Street, Glasgow G4 0NR, UK

G. G. Skellern

Correspondence: G. G. Skellern, Department of Pharmaceutical Sciences, SIBS, University of Strathclyde, 27 Taylor Street, Glasgow G4 0NR, UK

Acknowledgement: The authors wish to thank A. G. J. Daas for the statistical analysis and Miss Valerie Lejeune and Miss Allison Reid for the preparation of the text.

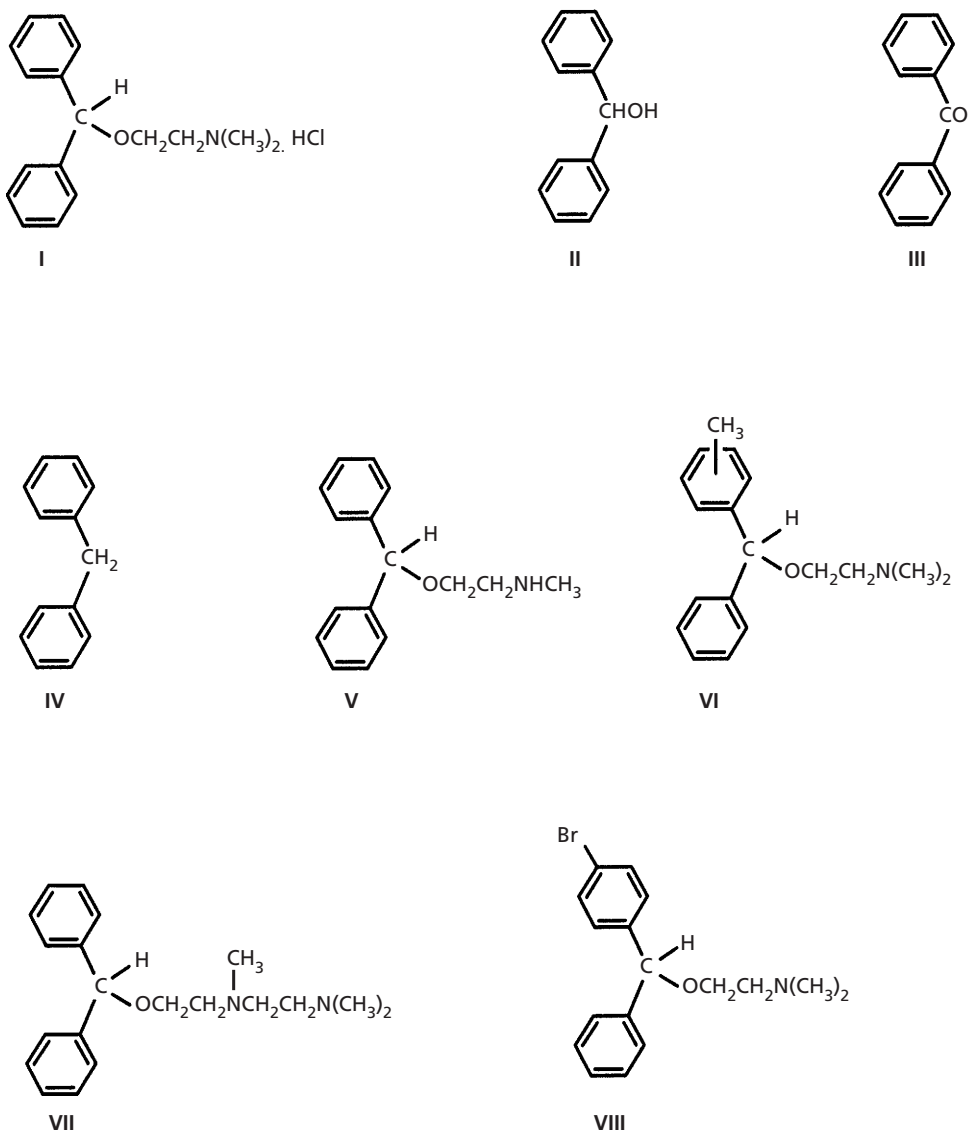

Figure 1 Structural formulae of diphenhydramine hydrochloride (**I**) and its potential impurities (**II**–**VIII**).

Materials and Methods

Solvents and reagents

Analytical-grade methanol, chloroform, dichloromethane, diethylamine, sulfuric acid, sodium lauryl sulfate, sodium dihydrogen phosphate dihydrate, sodium hydroxide and hydrochloric acid were supplied by Merck (Darmstadt, Germany). HPLC-grade acetonitrile was supplied by Merck and analytical-grade triethylamine was supplied by Fluka (Buchs, Switzerland).

Diphenhydramine hydrochloride was obtained from two manufacturers, Shanghai Fourth (Shanghai, China) and Recordati (Milan, Italy); impurities **II**–**VIII** were supplied by Recordati.

Thin-layer chromatography (TLC)

Commercial TLC pre-coated glass plates (20 × 20 cm), 0.25 mm layer thickness, were coated with silica gel HF_{254} (Alltech, Tempemars, France) and silica gel K6 (60 Å) (Whatman, NJ).

Mobile phase 1 consisted of chloroform–methanol–diethylamine (80:20:1, v/v). Mobile phase 2 consisted

of dichloromethane–methanol–diethylamine (80:20:1, v/v). The Chromatograms were developed at room temperature over a path of 10 cm. A sulphuric acid spray reagent was used to locate the analytes.

Related substances
Individual standard solutions (0.5 mg mL^{-1}) of impurities **II**–**VIII** and diphenhydramine hydrochloride were prepared in acetonitrile. Volumes (1 mL) of each solution of impurity were transferred to a 10-mL volumetric flask, after which 50 mg of diphenhydramine hydrochloride was added and the flask was filled to volume with acetonitrile. This produced a standard mixture containing diphenhydramine hydrochloride (5 mg mL^{-1}) and impurities (0.05 mg mL^{-1}) present at their limiting concentrations. Test solutions of diphenhydramine hydrochloride samples from each manufacturer were prepared by dissolving 100 mg of diphenhydramine hydrochloride in 10 mL of acetonitrile (10 mg mL^{-1}solutions).

Liquid chromatography

The liquid chromatographic system consisted of a P1000XR pump, an AS1000XR automatic injector, a UV150 variable wavelength detector (UV-VIS) and a model SP4400 integrator (all Thermo Separation Products, Orsay, France). pH measurements of the mobile phases were made with a Mettler InLab 419 pH meter and Delta 350 recorder (Mettler, Greifensee, Switzerland). Determination of the optimum wavelength of detection for the analysis of all the substances was performed using a Waters 996 photodiode array detector with Millennium software package (Waters, MA) using system 2 as described below.

For liquid chromatographic system 1, the mobile phase was acetonitrile-distilled water–triethylamine (100:100:1, v/v), which was delivered at 0.6 mL min^{-1}. Samples were injected through a fixed-volume (20 μL) loop onto a Hypersil BDS cyano-propylsilyl silica gel 5 μm (250 × 4.6 mm; Southern Shandon, Cheshire, UK) column. The detection wavelength was 254 nm. For system 2, the mobile phase was a mixture of 20 volumes of a 78-g L^{-1} solution of sodium dihydrogen phosphate dihydrate (adjusted to pH 6.0 with 10.5 M sodium hydroxide), 40 volumes of distilled water and 40 volumes of acetonitrile; the mixture contained 14.4 g L^{-1} of sodium lauryl sulfate and was delivered at 1.5 mL min^{-1}. Samples were injected through a fixed-volume (20 μL) loop onto a Lichrospher RP select B-C8, 5 μm (250 × 4.6 mm; Interchim, Montlucon, France) column. The detection wavelength was 225 nm.

Related substances
A test solution was prepared by accurately weighing diphenhydramine hydrochloride (100 mg) into a 10-mL volumetric flask and making up to volume with acetonitrile–water (40:60, v/v). Reference solutions were prepared by diluting a sample (1 mL) of the test solution to 100 mL with acetonitrile–water (40:60, v/v). Standard solutions (0.1 mg mL^{-1}) of impurities **II**–**VIII** and diphenhydramine hydrochloride (**I**) were prepared by dissolving 1 mg of each substance in 10 mL of acetonitrile–water, (50:50, v/v).

A mixture of **I** and each of the impurities (**II**–**VIII**), at their limiting concentrations, was prepared by transferring 1-mL volumes of each solution of impurity (0.1 mg mL^{-1}) to a 10-mL volumetric flask, after which 10 mg of **I** was added and the flask was made up to volume with acetonitrile–water (50:50, v/v). A mixture of **I** and each impurity at equal concentrations (0.01 mg mL^{-1}) was prepared by diluting 1 mL of each standard solution to 10 mL with acetonitrile–water (50:50, v/v).

Linearity and repeatability
Linearity and repeatability were determined from six injections of five solutions in a range of 60–140 % of the limiting concentration of the test solution. The solutions were prepared from two stock solutions of **I** prepared with acetonitrile–water (40:60, v/v). The test solution of **I** (10 mg mL^{-1}) was diluted with acetonitrile–water (40:60, v/v) to produce a 0.1-mg mL^{-1} solution. Samples of this solution were diluted to 10 mL, to produce 0.06-mg mL^{-1} and 0.08-mg mL^{-1} solutions. A second solution of **I** was prepared by dissolving 10 mg in 50 mL of acetonitrile–water (40:60, v/v). Samples of this solution were diluted to 10 mL to yield 0.120-mg mL^{-1} and 0.14-mg mL^{-1} solutions.

Stability of diphenhydramine hydrochloride

To evaluate the stability of a test solution of **I**, a sample (450 μL) was exposed to daylight over a 48-h period at room temperature. Other portions (450 μL) were mixed either with 0.1 M sodium hydroxide (50 μL) or 0.1 M hydrochloric acid (50 μL). Each solution was then analysed with liquid chromatograph system 2.

Limits of quantification and detection

The limit of quantification was determined from chromatograms of an injection of a solution which produced a response ten times that of the maximum variation in

baseline noise. The limit of detection was calculated from the limit of quantification (3 times the baseline noise).

Experimental design

The following four mobile-phase chromatographic variables were set at both high and low levels, covering the range within which robustness had to be established: concentration of acetonitrile (CH_3CN) as organic modifier; pH; buffer salt concentration; ion-pair reagent molarity.

This approach is similar to one previously used to test the robustness of a liquid chromatographic method for amoxicillin (Yongxin et al 1997). The low and high levels investigated are listed in Table 1, together with a central level. The levels are respectively coded -1, $+1$ and 0. These values correspond approximately to the adjustment which may be encountered during method transfer. The design of the applied full factorial method together with the statistical analysis of the measured response variables and the multivariate regression calculations were performed using statistical software (SAS/STAT 1990). The Pareto chart and response surface plots were produced with spreadsheet software (Microsoft Excel 1994).

Results and Discussion

TLC

The current Ph. Eur. monograph (*European Pharmacopoeia* 1997) on diphenhydramine hydrochloride de-

Table 1 Chromatographic parameters selected, and their ranges tested to assess the robustness of the method, for liquid chromatographic system 2 (nominal values corresponding to -1, 0 and $+1$).

Chromatographic parameter	Code	Low value (-1)	Central value (0)	High value ($+1$)
CH_3CN (% v/v)	A	35	40	45
pH of the buffer	B	5.5	6.0	6.5
Buffer salt concentration (M)	C	0.05	0.10	0.15
Ion-pair reagent[a] molarity (M)	D	0.04	0.05	0.06

[a]Sodium lauryl sulfate. This implies $2^4 = 16$ combinations for a full-fraction factorial design. The 16 combinations were permutated randomly and one measurement per combination was carried out. An additional measurement was carried out with all parameters set at a central level.

Table 2 R_f values of diphenhydramine hydrochloride (**I**) and impurities **II**–**VIII** using two types of silica gel stationary phase.

Substance	R_f value ($\times 100$)	
	Silica gel HF_{254}	K6 (60 Å)
Impurity **II**	81	77
Impurity **III**	Not detected	Not detected
Impurity **IV**	Not detected	Not detected
Impurity **V**	31	24
Impurity **VI**	47	31
Impurity **VII**	18	16
Impurity **VIII**	50	29
Diphenhydramine hydrochloride (**I**)	51	31
Mixture	27, 45, 85	22, 31, 79

Stationary phase: silica gel HF_{254} or K6 60 Å plate (layer thickness 250 μm) (20 × 20 cm). Eluting solvent: chloroform–methanol–diethylamine (80:20:1, v/v). Detection: sulfuric acid spray, followed by heating at 120°C for 15 min.

scribes, for the control of related substances, a TLC method to control each detected impurity at a level of 1%. However, it was shown that the method was insufficiently discriminatory to separate all the potential impurities of diphenhydramine hydrochloride (Table 2).

Separation was performed on two types of silica-gel stationary phase, silica gel K6 (60 Å) and silica gel HF_{254} plates. Silica gel K6 does not contain fluorescent indicator and silica HF_{254} does not contain calcium sulfate binder. Detection of analytes was achieved using a sulfuric acid spray, which produced yellow spots after the plates were heated at 120°C for 15 min. However, the selectivity of this method was not adequate with either type of stationary phase (Table 2). Moreover, impurities **III** and **IV** were not detected and mobile phase 1 was unable to separate any of the seven potential impurities of diphenhydramine hydrochloride, when applied as a mixture. The impurities in the mixture were present at their limiting concentrations (0.05 mg mL^{-1}).

The composition of the eluting solvent was modified to replace chloroform with dichloromethane (mobile phase 2). The volumes of each solvent were unchanged and the chromatography was repeated, but there were no changes in the selectivity of the method. Five samples of diphenhydramine hydrochloride, from two different sources, were examined using experimental conditions modified from the Ph. Eur. monograph for diphenhydramine hydrochloride (*European Pharmacopoeia* 1997) and no impurities were detected.

Using the conditions described for the related substances test in the Ph. Eur. monograph (*European Pharmacopoeia* 1997), only three spots appeared in the chromatogram of a standard mixture of diphenhydramine hydrochloride and impurities, present at their limiting concentrations. One spot corresponded to impurity **V**, another to impurity **II** and the third was a combination of impurity **VI**, impurity **VIII** and diphenhydramine hydrochloride. Since impurities **III** and **IV** were not detected and two impurities had similar R_f values to diphenhydramine hydrochloride (impurities **VI** and **VIII**), it was concluded that the TLC method was not able to adequately control the potential impurities of diphenhydramine hydrochloride.

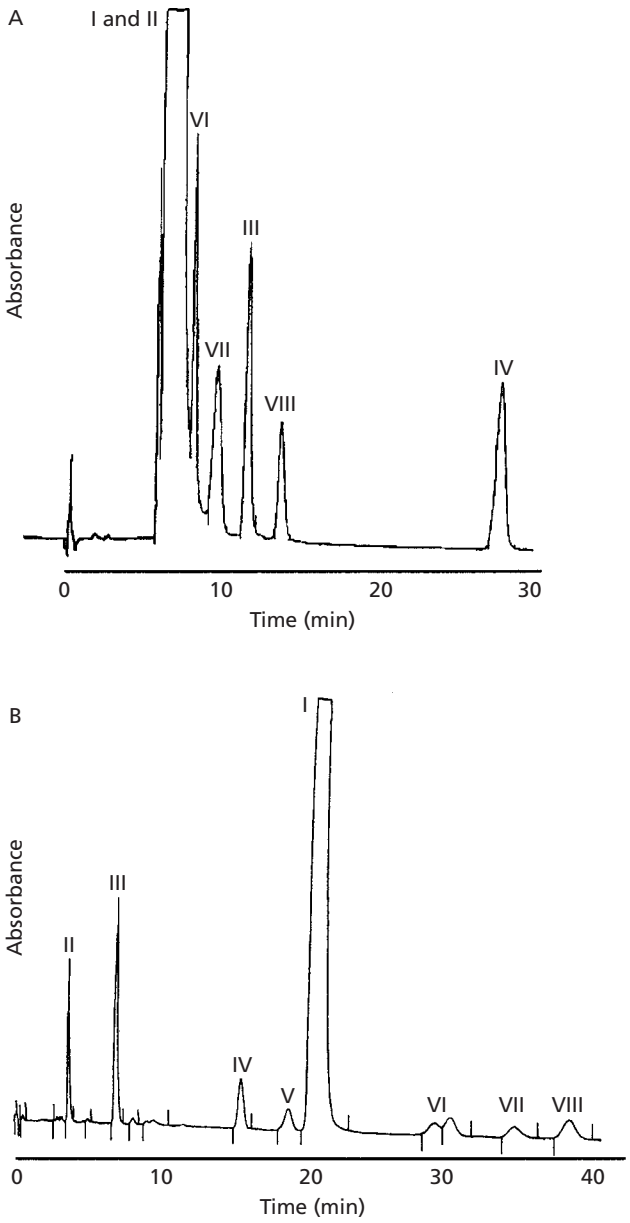

Figure 2 Liquid chromatogram of a solution of diphenhydramine hydrochloride (**I**) containing seven potential impurities at 1% of the diphenhydramine hydrochloride concentration. Experimental conditions: A, liquid chromatographic system 1; B, liquid chromatographic system 2.

Liquid chromatography

Two liquid column chromatographic methods (system 1 and system 2) for the control of impurities were compared.

Liquid chromatographic system 1, which was a modification of the method described for the assay of diphenhydramine hydrochloride in the *United States Pharmacopeia* (1995), produced insufficient separation of the impurities from diphenhydramine to control all the impurities at the 1% level. By changing the composition of the mobile phase to contain acetonitrile–water–triethylamine (350:650:4 v/v), separation of all the impurities, from each other and from diphenhydramine

Table 3 Relative response factors and relative retention times of the impurities of diphenhydramine hydrochloride (**I**) at the detection wavelength of 225 nm (relative to **I**) using liquid chromatographic system 2.

Impurity	Relative response factor	Relative retention time
II	1.02	0.25
III	0.87	0.40
IV	1.42	0.79
V	0.99	0.95
VI[a]	1.36	1.41,1.43
VII	1.27	1.65
VIII	0.74	1.82

[a]Mixture of structural isomers.

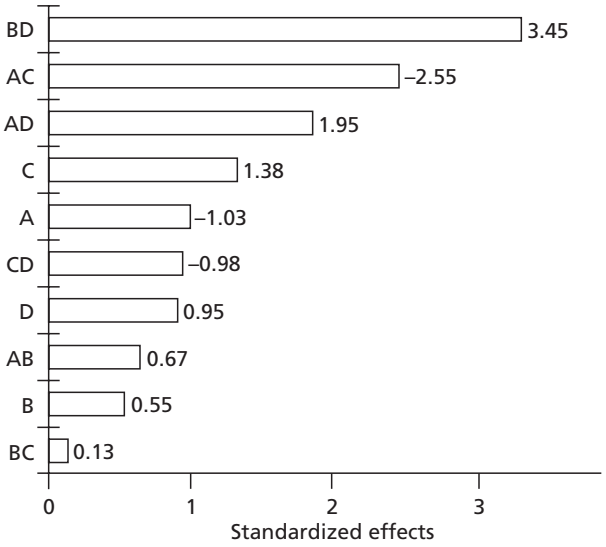

Figure 3 Pareto chart for selectivity between diphenhydramine hydrochloride (**I**) and impurity **V** ($\alpha_{I\text{-}V}$). A, acetonitrile content (% v/v); B, pH of the buffer; C, buffer salt concentration (M); D, ion-pair reagent (sodium lauryl sulfate) molarity (M).

hydrochloride (**I**), was achieved with a solution containing equal concentrations of each substance. However, when these impurities were added at a level of 1 % to a test solution of **I**, impurity **II** co-eluted with **I** and impurities **V** and **VI** were not resolved from the principal peak (Figure 2A). Thus it was concluded that the method was not satisfactory.

Liquid chromatographic system 2, however, separated all the impurities from each other and from **I**, even when present at the limiting level (1 %) (Figure 2B). This system was thus fully validated.

The two peaks, using liquid chromatographic system 2, in the chromatogram of a solution of impurity **VI** (Figure 2B) were concluded to be due to the structural isomers of this compound.

Table 4 Full-fraction factorial design and results.

Run	CH_3CN (A)	pH (B)	Buffer (C)	Ion-pair (D)	Retention time (min)		
					Compound **I**	Compound **V**	$\alpha_{I\text{-}V}$
1	−1	1	−1	1	17.2	15.2	2.05
2	1	1	−1	−1	16.5	15.2	2.28
3	−1	1	1	1	71.9	65.9	6.04
4	−1	−1	−1	−1	NA	NA	NA
5	1	1	−1	1	67.1	62.8	4.30
6	1	−1	−1	−1	12.4	11.1	1.26
7	−1	1	1	−1	47.3	41.9	5.37
8	−1	1	−1	−1	13.0	11.9	1.11
9	0	0	0	0	23.9	21.9	1.86
10	−1	−1	1	−1	NA	NA	NA
11	1	−1	1	1	42.1	39.8	2.31
12	−1	−1	−1	1	55.6	52.3	3.29
13	1	1	1	−1	16.7	15.7	0.99
14	1	1	1	1	40.1	36.7	3.48
15	1	−1	1	−1	47.8	43.7	4.09
16	−1	−1	1	1	57.1	54.9	2.28
17	1	−1	−1	1	17.4	15.8	1.63

NA, not able to obtain a stable baseline.

Validation

A detection wavelength of 225 nm was selected since this wavelength produced similar relative response factors for each substance (Table 3). However, since the detector responses for each impurity were not within an 80–120 % range of the response for diphenhydramine hydrochloride, the relative response factors were used to calculate corrected peak areas.

Repeatability of injection was demonstrated for solutions of diphenhydramine hydrochloride in a concentration range of 0.06–0.14 mg mL^{-1}, representing 0.6–1.4 % of the test solution concentration (10 mg mL^{-1}). Relative standard deviations were acceptable (0.20–1.58 %, from the highest to the lowest concentration of diphenhydramine hydrochloride, respectively).

Linearity between detector response, recorded as peak area, and diphenhydramine hydrochloride concentration ($y = 4.76 \times 10^7 x - 1.05 \times 10^5$, $r^2 = 0.9995$) was confirmed within the range 0.6–1.4 % of the test solution concentration (10 mg mL^{-1}).

The limit of quantification was determined from the concentration of diphenhydramine hydrochloride which produced a signal-to-noise ratio of 10 : 1 and was found to be 0.55 μg mL^{-1}. The detection limit, defined as the concentration producing a signal-to-noise ratio of 3 : 1, was calculated to be 0.17 μg mL^{-1}.

Chromatograms of acidic and alkaline solutions of **I**

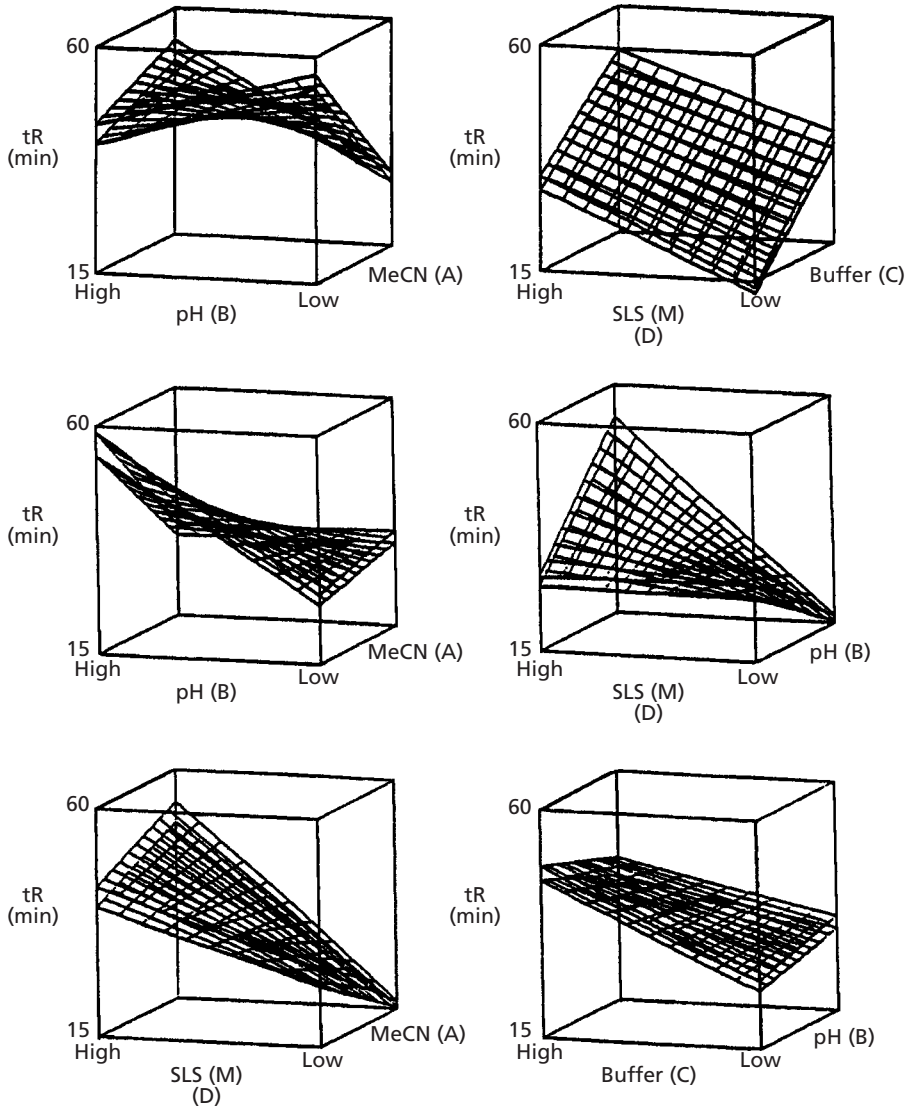

Figure 4 Estimated response surface plots for the retention times (tR) of diphenhydramine hydrochloride (**I**) and impurity **V**. A, acetonitrile (MeCN) content (% v/v); B, pH of the buffer; C, buffer salt concentration (M); D, ion-pair reagent (sodium lauryl sulfate; SLS) molarity (M).

and solutions of **I** exposed to daylight for 48 h at room temperature, showed no significant changes in the impurity profiles. Thus, diphenhydramine hydrochloride did not appear to undergo degradation under these conditions.

The composition of the mobile phase was altered to determine the susceptibility of the separation of diphenhydramine hydrochloride from impurity **V** to small variations in the mobile phase composition. The buffer salt concentration, sodium lauryl sulfate molarity, acetonitrile content and the pH of the mobile phase were adjusted. A solution of diphenhydramine hydrochloride and impurities **II**–**VIII**, present at their limiting concentrations, was injected under each set of conditions.

The measured response variables were the retention times of **I** and impurity **V**. The results are listed in Table 4, together with the calculated selectivity $\alpha_{I\text{-}V}$ values, which are the difference between the retention times of I and IV.

The Pareto chart, using the data in Table 4, for the selectivity between **I** and **V** is shown in Figure 3. This chart is not standardized because the data did not allow for an estimation of the residual error (for which at least two measurements per combination would be necessary). Only first-level interactions are considered in this analysis (i.e. only a combination of two parameters), with the largest effects appearing at the top of the scale and the smallest one at the base. Although this cannot be confirmed statistically, it may reasonably be assumed that the largest estimated effects are significant and that the smallest are not significant and are merely due to random effects.

It can be seen that the interaction between pH (B) and ion-pair reagent molarity (D) had the largest influence on the separation between **I** and **V**, although the two parameters individually were less important. This means that increasing both the ion-pair reagent molarity and the pH had a more pronounced effect on the separation. It can also be seen that increasing the buffer salt concentration (C) could positively influence the separation, but this effect would be negated if the acetonitrile concentration (A) was increased at the same time.

To obtain an impression of the retention times as a function of the controlled parameters, response surface plots (Figure 4) were generated for each possible combination of two parameters. A very striking feature of these plots was their large dynamic range, 12.7–58.8 min, whereas the distance between the two surfaces was always fairly small.

One of the interesting features of these plots was the importance of some of the interactions, notably with acetonitrile (A), causing the surfaces to be markedly curved or saddle-shaped. Considering the marked influence of first-level interactions on the retention times, it is highly probable that higher degrees of interactions also play a role. Small changes in the composition of the mobile phase, however, will not have an important influence on the selectivity of the method. Thus liquid chromatographic system 2 was considered robust, since the surfaces in the response surface plots never overlapped. A minimum resolution value of 1.5 between diphenhydramine hydrochloride (**I**) and impurity **V** is recommended.

Impurity profiles of samples of diphenhydramine hydrochloride from two different sources

The impurity profiles of five different diphenhydramine hydrochloride samples from two manufacturers were compared (Table 5). The impurity profiles significantly differed between the manufacturers, with one manufacturer's samples containing only one impurity (impurity **V**) and another manufacturer's samples containing three impurities (impurities **II**, **V** and **VI**). Only one peak appeared in the impurity-**VI** region in the chromatograms of two samples of diphenhydramine hydrochloride, hence it was concluded that only one of the structural isomers of impurity **VI** was present in those samples. Furthermore, the sum of impurities differed between the manufacturers' products – one product contained less than 0.07% m/m whilst the second manufacturer's product contained around 1% m/m.

Since impurity **VI** was not separated from diphenhydramine hydrochloride using the existing TLC method (Table 2) prescribed by the *European Pharmacopoeia* (1997) monograph for the control of related substances, the HPLC method we developed (liquid chromatographic system 2) could replace the current TLC method.

Table 5 Impurity profiles of batches of diphenhydramine hydrochloride

Sample	Impurity (% m/m)							
	II	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	Total
D1	–	–	–	0.07	–	–	–	0.07
D2	–	–	–	0.06	–	–	–	0.06
D3	–	–	–	0.06	–	–	–	0.06
D4	0.02	–	–	0.13	0.63	–	–	0.78
D5	0.02	–	–	0.13	0.85	–	–	1.00

References

European Pharmacopoeia 3rd Edition (1997) Council of Europe, 67075 Strasbourg Cedex, France, Monograph No. 0023

Microsoft Excel Analysis Toolpak. Version 5.0 (1994) Microsoft Corporation, Cambridge, MA

SAS/STAT User's guide (1990) Version 6, Fourth edition, Vol 2 SAS Institute Inc, Cary, NC

United States Pharmacopeia XXIII (1995). USP Convention Inc. Rockville, USA, p. 532

Yongxin, Z., Augustinjs, J., Roets, E., Hoogmartens, J. (1997) Robustness test of the liquid chromatographic method for the assay of amoxicillin. *Pharmeuropa* **9**: 323–327