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Validation of high-performance liquid chromatographic methods on two silica base-deactivated reversed phases for the determination of chloroprocaine and bupivacaine[1]

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Abstract

The separation by HPLC of basic drugs on silica-based reversed phases remains a major problem because of the interaction between the residual silanol groups of the silica and the amino function of the drug. This paper describes the validation of HPLC methods for the determination of two injectable solutions of basic drugs (two local anaesthetics, chloroprocaine and bupivacaine), with two commmercial base-deactivated reversed phases. These columns improve the chromatographic performances without adding a blocking agent to the mobile phase. With a simple aqueous–organic mobile phase, these base-deactivated reversed phases give high theoretical plate numbers (N) and small tailing factors. The optimized methods show good linearity, precision (RSD < 2%) and accuracy (bias < 2% for dosage forms). The limits of detection and quantitation are lower than the maximal accepted limits for impurities. These methods are currently in routine use in stability studies.

Keywords: Basic drugs; Local anaesthetics; Reversed-phase chromatography; Silica base-deactivated column; Validation

1. Introduction

Basic compounds have great importance in many fields of application such as the environment, farm produce and the pharmaceutical industry. In the last case, it is important to note that more than 80% of products are basic compounds and possess one or more amino functions [1] such as opianalgesics, local anaesthetics and amphetamines. Nowadays, HPLC using reversed-phase columns is the method of choice for analyses of these compounds. However, this separation still remains a difficult problem because of the interaction between amino functions and free residual silanol groups of the silica. This phenomenon induces important peak tailing and thus reduces the chromatographic performance. Therefore, for routine analyses, it is difficult to carry

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out a precise determination of basic compounds in order to validate a method as required [2–4].

Since 1989, many silica base-deactivated reversed phases have been made commercially available in order to improve the chromatographic performance in the separation of basic compounds [5]. These columns show high efficiency and a small asymmetry factor, with a simple mobile phase consisting of an organic solvent and an aqueous buffer, and without the addition of a blocking agent such as tertiary or quaternary amines [6–9]. Furthermore, the simple composition of the mobile phase enhances the lifetime of the column and the method is more rugged and more precise. Other supports have been developed for the separation of basic compounds, including polymer-based packings and alumina and zirconia packing materials [10–19]. These supports are more stable than silica material at pH > 8.

The aim of this study was to demonstrate the possibilities of using silica base-deactivated reversed phases instead of conventional C_{18} supports in analyses for basic compounds. This paper presents the validation [20–23] of HPLC methods on two of these supports for the determination of two local anaesthetics and their degradation products in aqueous solutions. These compounds, chloroprocaine and bupivacaine, are commercially available as injectable solutions in various concentrations. In our laboratory, the validated methods are now applied routinely for the stability studies required for drug registration and quality control. These preliminary results will be included in a overall project in which the behaviour and performance of various supports will be compared for the HPLC of several basic compounds.

2. Experimental

2.1. Materials and reagents

Chloroprocaine HCl was obtained from Orgamol (Evionnaz, Switzerland) and 4-amino-2-chlorobenzoic acid (ACBA) from Fluka (Buchs, Switzerland). Bupivacaine HCl was obtained from Schweizerhall (Basle, Switzerland) and 2,6-dimethylaniline (DMA) from Fluka. Stock solutions of these compounds were prepared in water purified by using a Milli-Q RG system (Millipore, Milford, MA). The structures of these compounds are shown in Fig. 1.

HPLC grade acetonitrile was supplied by Merck (Darmstadt, Germany) and was used without further purification. All compounds used to prepare buffer solutions were purchased from Fluka.

All the injectable solutions of chloroprocaine (5 mg ml^{-1}) and bupivacaine (1.25 mg ml^{-1}) were a gift from Sintetica (Mendrisio, Switzerland).

2.2. Instrumentation

HPLC analyses were performed at a flow rate of 1 ml min^{-1} using a Merck–Hitachi L-6200 A pump (Darmstadt, Germany), a Merck–Hitachi As-2000A automatic sample injection system, a Merck–Hitachi L-5025 oven fixed at 30°C and a Merck–Hitachi L-4500 UV–Visible diode-array detector (DAD). Instrument control and data ac-

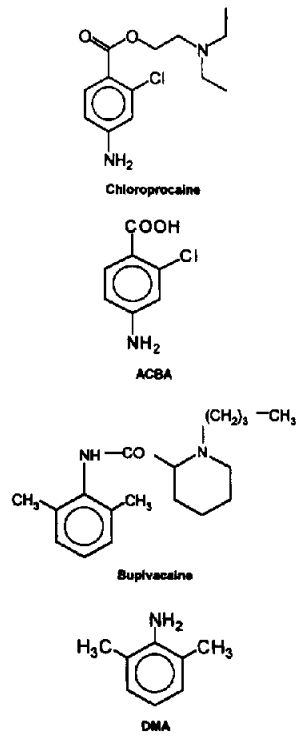

Fig. 1. Structures of the tested compounds.

Table 1
Concentrations of working standard solutions of the tested compounds

Compound	Stock standard solution (mg ml^{-1})	Concentration range (mg ml^{-1})
Chloroprocaine	5.00	0.08–0.12
ACBA	0.50	9.12×10^{-4}–3.6×10^{-3}
Bupivacaine	1.25	0.83–1.25
DMA	1.00	3.72×10^{-7}–3.13×10^{-3}

quisition were effected by a Dell 433/L microcomputer with a Merck–Hitachi D-6000 interface.

2.3. LC conditions

Chloroprocaine and ACBA were analysed on a 5 μm Supelcosil LC-ABZ column (150 × 4 mm i.d.) (Supelco, Bellefonte, PA) and a 5μm Nucleosil 100 C_{18} column (125 × 4 mm i.d.) (Macherey–Nagel, Düren, Germany). The mobile phase was acetonitrile–acetate buffer solution (pH 4.5, 0.1 M) (20:80, v/v). A 1 l volume of acetate buffer was prepared by dissolving 14.77 g of sodium acetate trihydrate and 1.145 ml of acetic acid in water. The pH was adjusted to 4.5 with 1 M HCl. Volumes of 10 μl of the chloroprocaine solution were injected and the detector was set at 288 nm.

Bupivacaine and DMA were analysed on a 5 μm Nucleosil 100 C_{18} AB column (125 × 4 mm i.d.) (Macherey–Nagel). The mobile phase was acetonitrile–phosphate buffer solution (pH 7.0, 0.05 M) (50:50, v/v). A 1 l volume of buffer solution was prepared by dissolving 3.40 g of KH_2PO_4 and 4.35 g of K_2HPO_4 in water. The pH was adjusted to 7.0 with 1 M NaOH. Volumes of 20 μl of the bupivacaine solution were injected and the detector was set at 263 nm.

2.4. Preparation of standard and sample solutions

Stock standard solutions were obtained by dissolving appropriate amounts of compounds in water to give the concentrations reported in Table 1, except for DMA, which was dissolved in acetonitrile–water (50:50, v/v). These solutions were stored at 4°C for a maximum of 1 month.

For the method validation, the linearity, precision and accuracy were determined on standard aqueous samples and on reconstituted samples. The linearity of the method for chloroprocaine and bupivacaine was confirmed, using classical statistical tests on five calibration standards covering the range 80–120% of the target concentration. Each concentration was prepared in duplicate and analysed in triplicate. For this purpose, working standard aqueous solutions of chloroprocaine and bupivacaine were prepared by diluting the stock standard solutions with water in appropriate volumetric flasks. The reconstituted solutions of chloroprocaine and bupivacaine were also prepared by dilution in order to cover the same concentration range (80–120%).

For the linearity of ACBA and DMA, which are degradation products of chloroprocaine and bupivacaine, respectively, reconstituted solutions were spiked with known amounts of these two compounds so as to cover the concentration range reported in Table 1. The solutions were previously analysed to confirm, before spiking, the absence of degradation products. Working standard aqueous solutions of ACBA and DMA were prepared by diluting the stock standard solutions with water in order to cover the same concentration range (Table 1).

For the stability studies, the injectable solution of chloroprocaine was diluted with water by a factor of 50 in a volumetric flask. The injectable solution containing 1.25 mg ml^{-1} of bupivacaine was diluted by a factor of 1.2.

3. Results and discussion

3.1. LC conditions

In stability studies for pharmaceutical formulations, it is necessary to have a validated analytical method available. In this work, we used HPLC coupled with UV detection to separate two local anaesthetics and their degradation products (Table 1). This method is considered as the method of choice because of its simplicity and

Table 2
Chromatographic parameters required for validations

Parameter	Chloroprocaine	ACBA	2,6-Dimethylaniline	Bupivacaine
k'	0.70	1.55	2.10	6.65
Asymmetry factor	<1.30	<1.30	<1.35	<1.30
N	>8000	>6000	>8000	>9000
Resolution	>6		>14	

rapidity. Moreover, the use of base-deactivated silica supports, developed for the separation of basic compounds, allows one to simplify the mobile phase composition and to increase the analytical ruggedness. For the separation of chloroprocaine and bupivacaine solutions, we determined the optimal composition of the mobile phase by adjusting the pH, the nature of the buffer and its concentration and the ratio of acetonitrile (unpublished data).

This study was carried out with two silica base-deactivated columns. The chromatographic performances were almost identical for both compounds with the Supelco ABZ and the Macherey–Nagel AB columns. Therefore, in this paper we chose to present validation data in the Supelco ABZ column for the analysis of chloroprocaine solutions and on the Macherey–Nagel AB column for the bupivacaine solutions.

Under the specified conditions, we separated the drugs and their degradation products in less than 10 min with excellent efficiency and asymmetry factor and a resolution always higher than 6, as shown in Table 2. In the case of the asymmetry factor, As, we used the equation $As = W/2A$, where W is the width of the peak at 5% of its height and A is the distance from the front edge of the peak to the perpendicular at the peak maximum [22].

The chromatograms obtained with the optimized conditions of two standard solutions used for the validation are shown in Figs. 2 and 3. It can be seen that the deactivated, Supelcosil ABZ, designed for basic and acidic compounds, shows an excellent separation of chloroprocaine and ACBA without the addition of a blocking agent to the mobile phase. Using the method developed by Gill et al. [24], validated analyses carried out for this separation on conventional C_{18} columns need the addition of hexylamine to the mobile phase for efficient separation of the chloroprocaine peak. Furthermore, the peak of the ACBA was obtained with a high capacity factor and a relatively poor efficiency (as shown in Fig. 4). Hence, the limit of quantification was relatively high for the determination of this impurity (around 1% (w/w) of chloroprocaine). Work is in progress in our laboratory to compare various base-deactivated columns for the separation of acidic and basic compounds and to understand the interaction mechanisms of these supports better.

3.2. Method validation

For these two test solutions, we validated the chromatographic separations according to criteria described in the literature [22,23], such as selectivity, linearity, precision, accuracy and limits of detection and quantification for degradation products.

3.2.1. Selectivity

For all solutions, the selectivity was determined by injecting blank samples, placebos, standard solutions, injectable solutions and reconstituted formulations. No interfering peak was detected at the retention times of the drugs and degradation products. The peak shapes and the retention times of injectable solutions were similar to those obtained with standard solutions and reconstituted formulations.

3.2.2. Linearity

For bupivacaine and chloroprocaine, we verified the linearity of the method by injecting five

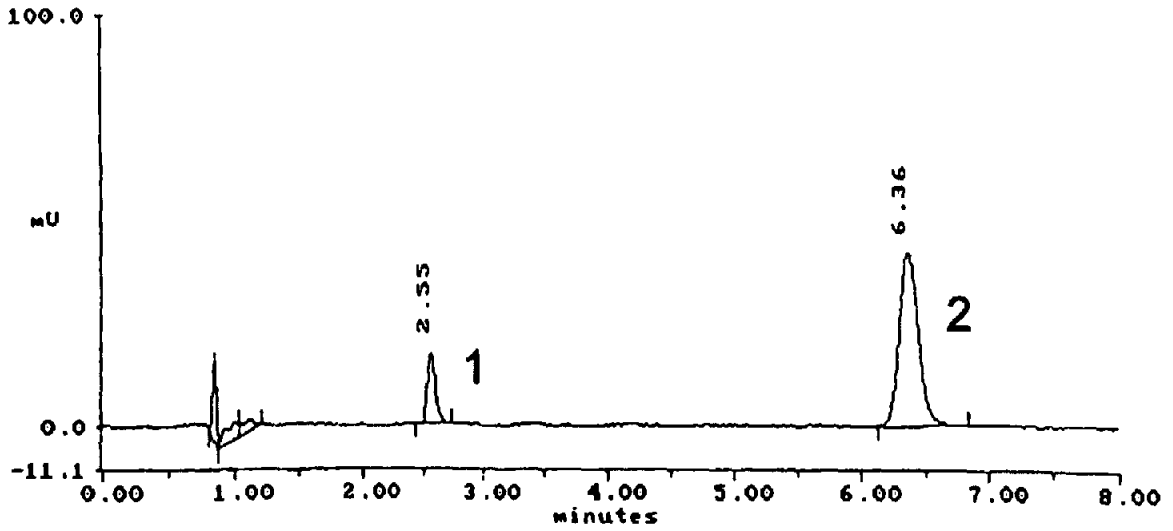

Fig. 2. Separation of bupivacaine (peak 2) and the degradation product (peak 1) 2,6-dimethylaniline. Separation conditions: injection, 20 μl of the bupivacaine solution on a deactivated column of Nucleosil 100 C_{18} AB (125 × 4 mm i.d.); mobile phase, acetonitrile–buffer solution (pH 7.0; 0.05 M) (50:50, v/v) at a flow rate of 1 ml min^{-1}; UV detection at 263 nm.

concentrations between 80 and 120% of the expected concentration. In this case, we reported the chromatographic peak area of the drug as a function of its concentration. For each compound, we carried out two calibration series, one with standard samples and the other with diluted reconstituted solutions. In order to simplify the description of the results, we present, in Table 3 only, data obtained with reconstituted solutions.

In all cases we showed, according to suitable statistical tests [23], that the intercept was not different from zero and the correlation coefficient was close to 1. Moreover, slopes obtained with standard and reconstituted solutions were not significantly different from each other. Hence the method does not show a matrix effect and an external standard solution, containing 100% of the expected drug, can be used to determine the unknown concentration in stability studies.

We used the same procedure for the degradation products. In this case, the lower limit corresponds to the limit of quantification and the upper limit corresponds to 4% (w/w) of the expected content of the drug. In the US Pharmacopeia XXIII, it is actually mentioned that chloroprocaine cannot contain more than 3% of ACBA and we therefore chose 4%, for both degradation products, as a maximum analytical limit. As for chloroprocaine and bupivacaine, the calibration curves obtained were linear, the intercept was not significantly different from zero and slopes of external standard and reconstituted solutions, with addition of ACBA and DMA were not significantly different (Table 3). Similarly, unknown concentrations of ACBA and DMA can be determined with an external standard solution containing 1.5% (w/w) of the expected drug content.

3.2.3. *Precision and accuracy*

The precision of the method was determined, as previously, with standard and reconstituted solutions. Six solutions containing 100% of the drug and 1.5% (w/w) of the degradation products were injected three times. This procedure was repeated on three days in order to obtain the between-run precision. As shown in Table 3, the precision expressed by the relative standard deviation is acceptable for drugs and degradation products and is not significantly different for standard and reconstituted solutions.

The accuracy was determined for all concentrations of the standard calibration curve. For each point, the experimental concentration was determined by recalculation with the external standard solution. In every case, the percentage recovery

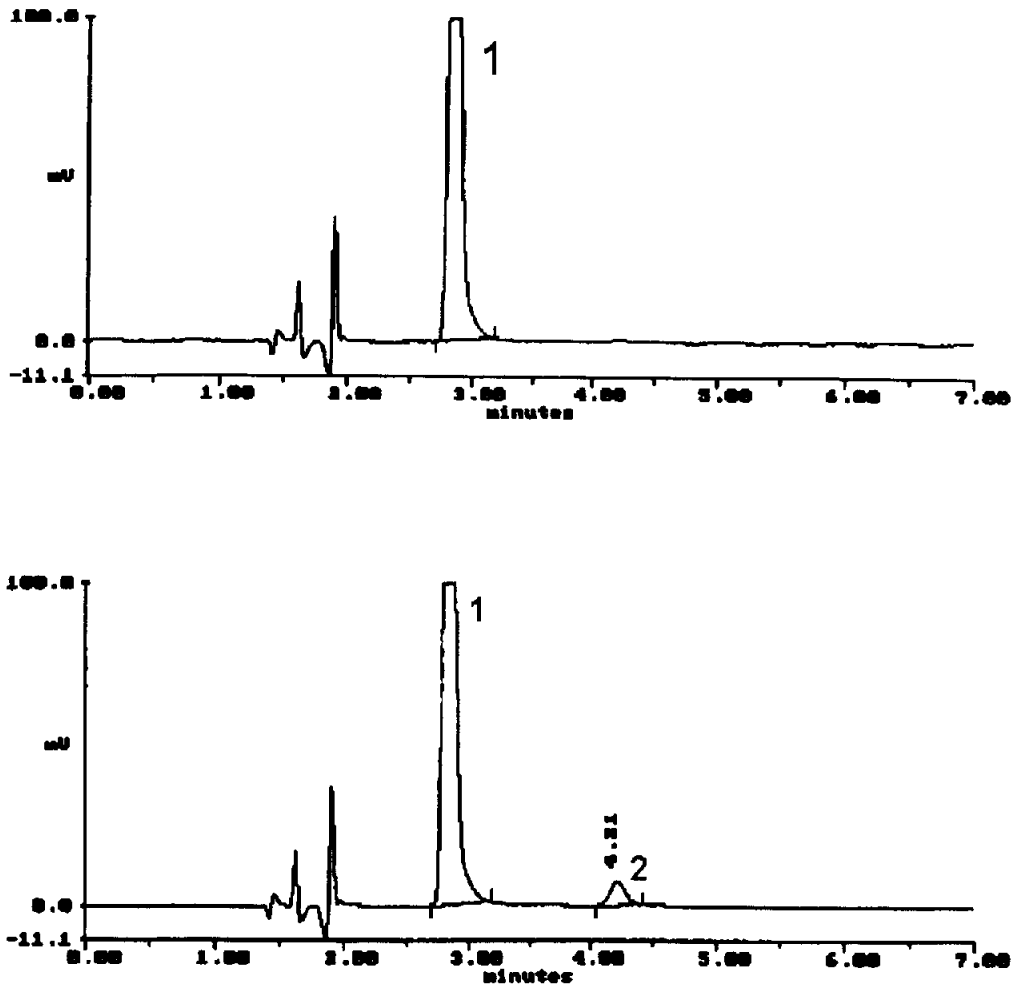

Fig. 3. Separation of chloroprocaine (peak 1) and the degradation product (peak 2) ACBA immediately after preparation (top) and after 12 months of storage at 35°C (bottom). Separation conditions: injection, 10 μl of the chloroprocaine solution on a deactivated column of Supelcosil LC-ABZ (150 × 4 mm i.d.); mobile phase, acetonitrile–buffer solution (pH 4.5; 0.1 M) (20:80, v/v) at a flow rate of 1 ml min^{-1}; UV detection at 288 nm.

was calculated and the accuracy was expressed by the bias (and the RSD of this bias) and determined by the difference (in %) between 100% and the percentage recovery. As shown in Table 3, the method is accurate in the linear dynamic range. The systematic error is below 2% for drugs and below 5% for degradation products.

3.2.4. Limits of detection and quantification

The limits of detection and quantification were determined for degradation products. These limits, obtained for signal-to-noise ratios of 3:1 and 10:1, respectively, are shown in Table 3. For ACBA and DMA, the limits of quantification correspond to 4×10^{-2}% (w/w) of chloroprocaine and 2.6×10^{-5}% (w/w) of bupivacaine, respectively. These values are clearly lower than required for stability studies.

These two limits were subsequently validated by the analysis of six samples known to be near or prepared at the limit of detection; the RSD for the limit of quantification was lower than 10% [25].

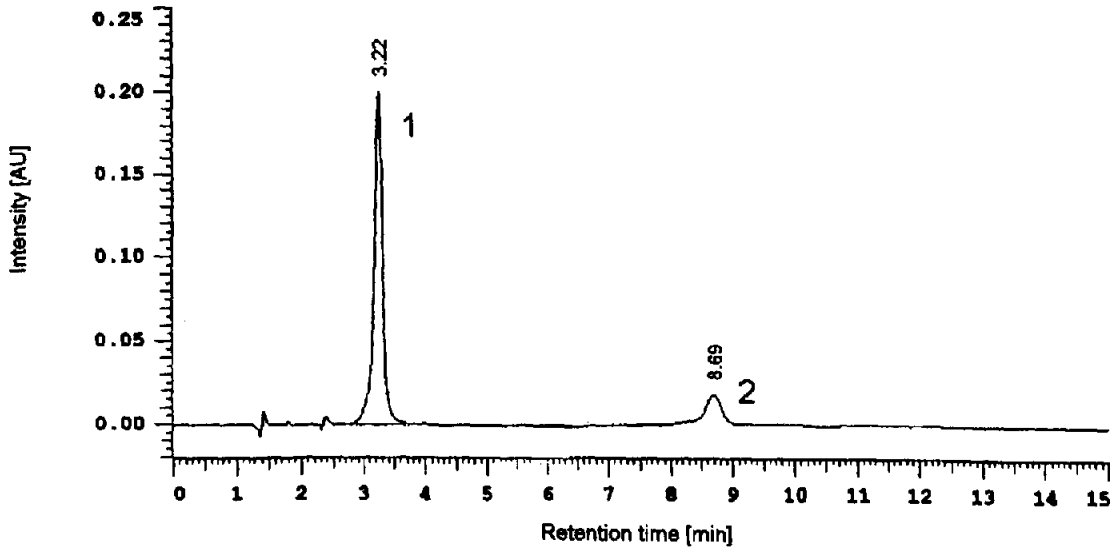

Fig. 4. Separation of chloroprocaine (peak 1) and the degradation product (peak 2) ACBA. Separation conditions: injection, 20 μl of the chloroprocaine solution on a column of Nucleosil 100 C_{18} (125 × 4 mm i.d.); mobile phase, methanol–water–80% phosphoric acid–*n*-hexylamine (150:850:5:7, v/v/v/v) at a flow rate of 1 ml min^{-1}; UV detection at 288 nm.

3.2.5. Stability studies

The procedures developed are currently used in our laboratory in stability programmes concerning the two injectable solutions of chloroprocaine and bupivacaine. Analyses are performed on many batches which are stored at various temperatures for several months, according to guidelines [26].

These tests indicated that solutions, stored at 4°C for more than 6 months do not show significant variations of the measured concentration. Thus, standards used for the quantitation and stored at 4°C for 1 month show satisfactory stability.

Stability studies are in progress and only partial results can be given here. Fig. 3 presents the chromatograms of chloroprocaine immediately after preparation (top) and after 12 months of

Table 3
Criteria of the validation for reconstituted solutions

Compound	Linearity		Repeatability RSD %	Reproducibility RSD %	Bias (%) (RSD (%))	Limit of detection (mg ml^{-1})	Limits of quantitation (mg ml^{-1})
	r^2	$y = a + bx$					
Chloroprocaine	0.9996	$5.51 \times 10^{-4} + 6.32 \times 10^{-6}x$	0.04	1.13	<1.7 (<0.5%)	ND[a]	ND
ACBA	0.9960	$-1.37 \times 10^{-4} + 1.06 \times 10^{-6}x$	2.26	2.43	<4.7 (<2.0)	2.74×10^{-4}	9.12×10^{-4}
Bupivacaine	0.9990	$7.16 \times 10^{-3} + 3.45 \times 10^{-6}x$	0.33	0.42	<1.7 (<0.5%)	ND	ND
2,6-Dimethylaniline	0.9998	$2.89 \times 10^{-4} + 4.28 \times 10^{-7}x$	1.77	1.99	<4.8 (<2.0%)	9.29×10^{-8}	3.72×10^{-7}

[a] ND = not determined.

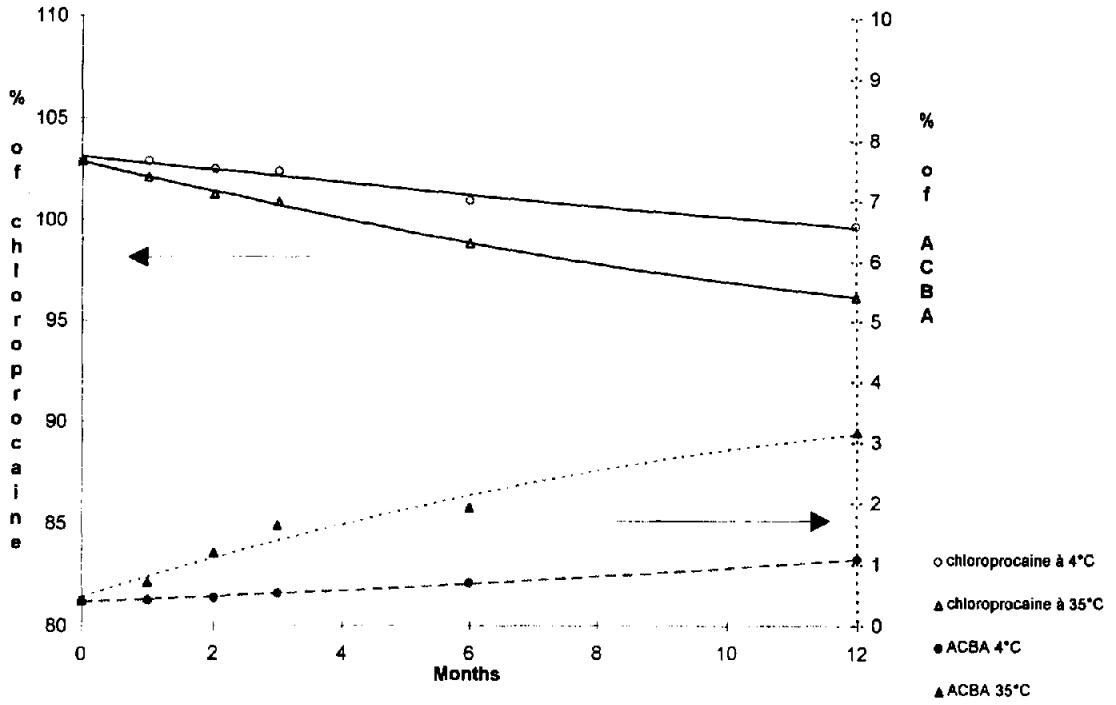

Fig. 5. Stability data for chloroprocaine solutions stored at 4 and 35°C for 12 months. Compound concentrations are given in % (w/w). ○, chloroprocaine at 4°C; △, chloroprocaine at 35°C; ●, ACBA at 4°C; ▲, ACBA at 35°C.

storage at 35°C (bottom). Fig. 5 shows the stability data obtained for two solutions of chloroprocaine stored at 4 and 35°C for 12 months. It can be seen that the presence of the degraded product, in this case ACBA, is clearly demonstrated at 35°C.

4. Conclusion

We have demonstrated that two columns packed with silica base-deactivated reversed phases gave excellent results for the separation of two basic drugs and their degradation products in comparison with conventional C_{18} or C_8 columns and without the addition of blocking agents to the mobile phase. The chromatographic performances of these columns allowed us to carry out validations of the analytical methods. The precision, accuracy and linearity were satisfactory and the limits of quantitation of the degraded products were lower than required for stability studies.

Work is in progress to study the behaviour and to compare the performances of these base-deactivated columns, commercially available from various manufacturers.

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