ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
25 (2001) 861–869

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Stability of stored methacholine solutions: study of hydrolysis kinetic by IP-LC

V. Acar *, J.J. Houri, M.D. Le Hoang, D. Pradeau, F. Guyon

Pharmacie Centrale des Hôpitaux de Paris — Agence Générale des Approvisionnements Médicaux, Service des Affaires Réglementaires et de Développement Analytique, 7, rue du Fer à Moulin, 75005 Paris, France

Received 10 October 2000; received in revised form 6 December 2000; accepted 29 December 2000

Abstract

Methacholine chloride is a powerful cholinergic bronchoconstrictor agent used during bronchial airway hyper-responsiveness diagnosis. Methacholine is susceptible to hydrolysis in aqueous solutions in acetic acid and β-methylcholine. In the present work, kinetics of hydrolysis with different solvents (water and phosphate-buffered saline (PBS) pH 7.4) at different temperatures have been studied using a newly developed high-performance liquid chromatography. At 4°C, kinetic determination of hydrolysis in methacholine chloride solutions (50 mg/ml) shows no hydrolysis in either aqueous or phosphate-buffered solutions over a 40-day period. At 30°C, concentration of unbuffered methacholine chloride solutions remained unchanged, but buffered methacholine chloride solutions have degradation up to 5.5% over a 40-day period. At 40°C, concentration of unbuffered methacholine chloride has degradation up to 5% and buffered methacholine chloride solutions have degradation up to 10% over a 40-day period. Methacholine chloride solutions are susceptibly to be used in hospital pharmacy at different concentrations. We have studied pH and osmolality for methacholine solutions prepared with different diluents potentially used in hospital pharmacies, i.e. deionized water, 0.9% NaCl and PBS pH 7.4. We have demonstrated that methacholine solutions prepared with deionized water at 50 mg/ml and diluted with PBS pH 7.4 from 5 to 40 mg/ml are isoosmotic and potentially available for inhalation tests to measure non-specific bronchial hyper-responsiveness. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Methacholine chloride; Hydrolysis kinetic; Ion-pair high-performance liquid chromatography; Method validation; Stability

1. Introduction

Methacholine chloride is known to be an efficient and powerful cholinergic bronchoconstrictor agent, currently used in bronchial airway hyper-responsiveness diagnosis [1–6]. Many studies have proposed different challenge methods using methacholine chloride [7,8]. However, the accuracy of using bronchoconstrictor response is dependent on the precise concentrations of methacholine chloride. Numerous researches have

* Corresponding author. Tel.: +33-1-46691340; fax: +33-1-46691420.
E-mail address: valerie.acar@pch.ap-hop-paris.fr (V. Acar).

PII: S0731-7085(01)00377-6

indicated that the stability of methacholine chloride solutions depends on various parameters: storage conditions [9], nature of dilution, i.e. 0.9% sodium chloride [9–12], PBS and sodium bicarbonate buffer [10]. Usually, test solutions ranging from 0.03 to 25 mg/ml have been used during a broncho-provocation challenge. These solutions are not available commercially. Dilutions must be extemporaneously and rapidly prepared from crystals to prevent any deliquescence of the drug [13,14]. Methacholine chloride possesses an ester moiety, which is susceptible to hydrolysis in aqueous solution, producing acetic acid and β-methylcholine and consecutively to induce an acidic pH. Different studies have shown that inhalation of acidic aerosols can induce bronchoconstriction in asthmatic subjects [15–17]. Moreover, several authors have reported that non-isotonic solutions can induce a bronchial hyper-responsiveness and/or a severe bronchoconstriction [18,19]. For these reasons, we have studied in a first time, the better conditions in term of stability to prepare bulk methacholine solutions. Several authors have reported different methodologies for methacholine chloride assay: colorimetric method [9], high performance liquid

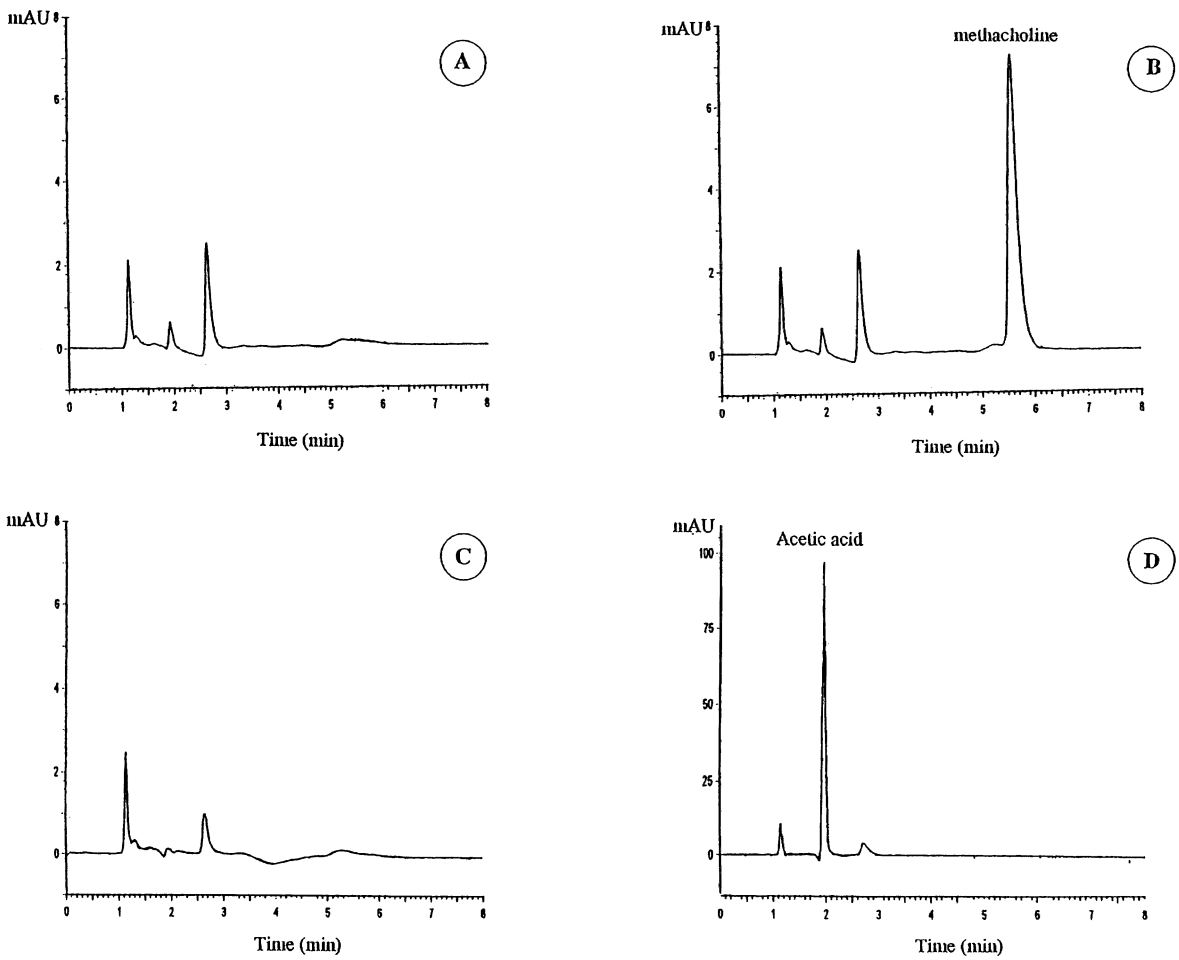

Fig. 1. Chromatograms resulting from the analysis of mobile phase (A), methacholine chloride solution in deionized water (250 μg/ml) (B), β-methylcholine solution in deionized water (5 μg/ml) (C) and acetic acid solution (520 μg/ml) (D). Detection wavelenght = 210 and 205 nm, respectively. Chromatography conditions are as described in Section 2.

Table 1
Accuracy in the assay determination of methacholine chloride

Day of analysis	Nominal concentration (mg/ml)	Concentration found (mg/ml)	Recovery (%)
1	50.89	50.975	100.168
	50.19	50.123	99.867
	49.98	50.217	100.475
	50.40	50.256	99.714
2	49.25	48.746	98.976
	51.01	50.173	98.360
	51.55	50.791	98.529
	49.98	49.980	100.000
3	50.11	49.560	98.903
	50.08	49.267	98.377
	50.21	49.585	98.757
	50.17	50.170	100.000

Table 2
One-way analysis of variance

Source of variation	Degrees of freedom	Sum of squares	Mean squares	*F*-ratio
Between days	2	1.142	0.571	1.660
Within days	9	3.097	0.344	
Total	11	4.239		

Table 3
Intra- and inter-day assay variability of the HPLC method for determining methacholine chloride concentrations

Day of analysis	Concentration found (mg/ml)	Mean (mg/ml)	S.D.	R.S.D. (%)
Intra-day variability ($n = 4$)				
1	50.98, 50.12, 50.21, 50.25	50.39	0.392	0.778
2	48.74, 50.17, 50.79, 49.98	49.92	0.857	1.717
3	49.56, 49.27, 49.58, 50.17	49.65	0.378	0.761
Inter-day variability ($n = 12$)				
	49.98		0.377	0.755

chromatography (HPLC) method [20], and most recently, capillary electrophoresis [21,22]. In the present work, we have developed and validated a new HPLC method to follow the stability of methacholine chloride dissolved in water or phosphate-buffered saline (PBS) pH 7.4 at 50 mg/ml and subjected to various temperatures: + 4, + 30, + 40°C at various times over a 40-day period. In a second time, we have measured two physico-chemical characteristics: pH and osmolality in function of methacholine chloride concentrations used in hospital pharmacies.

Table 4
The factors examined and their levels[a]

Factor	Levels		
	(−)	(+)	(0)
A. pH	2.25	2.75	2.50
B. Acetonitrile (%)	8.89	13.33	11.11
C. Sodium heptanesulfonate (g/l)	0.9	1.1	1

[a] The extreme levels are represented by (−) and (+) and the nominal ones by (0).

Table 5
Full factorial design for three factors and experimental measurements

Experiment number	Factors			Interactions				Experimental area
	A	B	C	AB	AC	BC	ABC	
1	−	−	−	+	+	+	−	88672
2	+	−	−	−	−	+	+	89042
3	−	+	−	−	+	−	+	87327
4	+	+	−	+	−	−	−	86181
5	−	−	+	+	−	−	+	89519
6	+	−	+	−	+	−	−	90079
7	−	+	+	−	−	+	−	89714
8	+	+	+	+	+	+	+	88991
Mean								88691.62
S.D.								1311.09

Table 6
Student t-test results[a]

	A	B	C	AB	AC	BC	ABC
Σy_{i-}	354 293	352 213	358 303	353 363	355 069	356 418	354 879
Σy_{i+}	355 232	357 312	351 221	356 162	354 455	353 107	354 645
a_i	−117.42	−637.42	885.25	−349.92	76.75	413.92	29.25
t-test	0.253	1.375	1.91	0.755	0.166	0.893	0.063
$= a_i/(\text{S.D.}/\sqrt{N})$							
Conclusion	NS	NS	NS	NS	NS	NS	NS

[a] $t = 2.36$, with 7 degrees of freedom, $P = 0.05$, NS, not significant.

2. Experimental

2.1. Chemicals

Methacholine chloride (acetyl-β-methylcholine chloride) was obtained from SERATEC (Epinay sur Seine, France) and stored at +4°C in its original container. Sodium chloride, potassium chloride, potassium dihydrogen phosphate, disodium hydrogen phosphate, triethylamine were purchased from Merck (Nogent sur Marne, France). Heptanesulfonic acid was purchased from Sigma (St Quentin, France). Phosphoric acid, Normapur, was purchased from Prolabo (Fontenay sous bois, France). Acetonitrile, HPLC grade, was purchased from Carlo Erba (Val de Reuil, France).

Water was deionized and filtered through a Milli-Q water purification system from Millipore (Molsheim, France).

2.2. Equipment

The liquid chromatography system consisted of Thermo Separation Product (TSP) P4000 pump, TSP AS 3000 auto sampler equipped with a 20 μl loop injector and the TSP UV 2000 detector (Les Ulis, France). The HPLC system was piloted by PC 1000 software (TSP). Detection wavelength was set at 210 nm. The Nucleosil C18 column (150 × 4.6 mm) was from Touzart et Matignon (Vitry sur Seine, France).

The pH content of methacholine chloride samples was measured using a pH meter (Methrom). The osmolality of methacholine chloride solutions

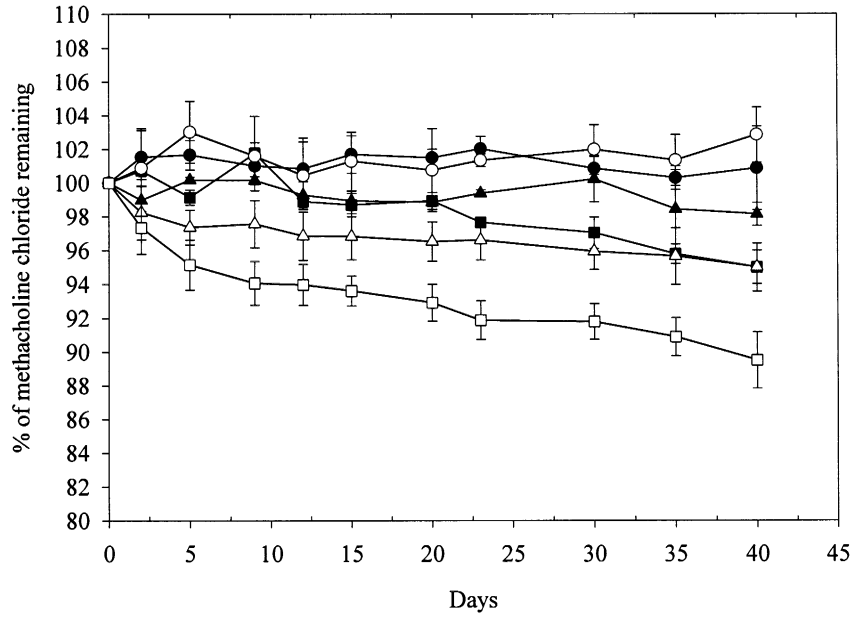

Fig. 2. Percent changes of methacholine chloride solutions, relative to initial concentration, dissolved either in deionized water or phosphate buffer pH 7 and subjected to various conditions of temperature for 40 days. H_2O, 5°C (●); H_2O, 30°C (▲); H_2O, 40°C (■); PBS 5°C (○); PBS 30°C (△); PBS 40°C (□). Values are the mean of three determinations.

was measured using an automatic osmometer (Roebling), with 300 mOsm/kg potassium chloride as standard.

2.3. Samples preparation

The bulk solutions of methacholine chloride (50 mg/ml) were freshly prepared by dissolving appropriate amounts in deionized water or PBS containing 1.86 mM potassium dihydrogen phosphate, 17.9 mM disodium hydrogen phosphate, 0.1 M sodium chloride adjusted to an apparent pH of 7.4 with 0.1 M phosphoric acid. The methacholine chloride solutions (50 mg/ml) were filtered through a 0.22 μm filter to prevent bacterial contamination. Methacholine chloride solutions were stored in rubber-sealed glass vials.

2.4. Stability study

Methacholine solutions were stored in temperature-controlled areas (5 ± 3; 30 ± 2 and 40 ± 2°C, mean ± range). At appropriate time intervals, samples were removed from each solution and methacholine concentrations were determined. The samples were assayed in triplicate.

2.5. Chromatographic conditions

Separation was carried out isocratically with the following solvent containing acetonitrile, 0.025 M disodium hydrogen phosphate, 0.01 M heptanesulfonic acid, 5.77 mM triethylamine, (pH 2.50) (11/89, v/v). The mobile phase was filtered under vacuum through a 0.45 mm GHP filter. Under these conditions and with a flow rate of 1 ml/min, the retention time of methacholine was 5.4 ± 0.07 min.

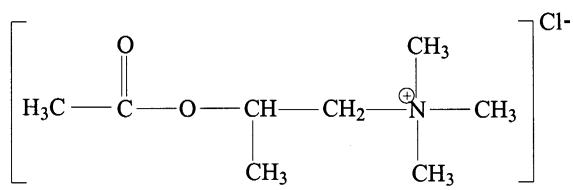

Fig. 3. Chemical structure of methacholine chloride.

Table 7
pH of methacholine solutions

Methacholine chloride concentration (mg/ml)	Unbuffered (deionized water diluent)	Unbuffered (0.9% sodium chloride diluent)	Buffered (phosphate buffer diluent)[a]
50	4.99	5.09	4.99
25	5.27	5.22	7.32
10	5.37	5.22	7.33
5	5.50	5.22	7.35
1	5.46	5.25	7.37
0.5	6.06	5.83	7.41
0.05	6.02	5.71	7.41

[a] Methacholine chloride solution was prepared at 50 mg/ml concentration in deionized water and diluted with PBS pH 7.4.

Table 8
Osmolality of methacholine solutions

Methacholine chloride concentration (mg/ml)	Unbuffered (deionized water diluent) (mOsm/kg)	Unbuffered (0.9% sodium chloride diluent) (mOsm/kg)	Buffered (phosphate buffer diluent) (mOsm/kg)[a]
50	459	758	459
25	229	521	363
10	94	377	287
5	49	331	260
1	10	295	241
0.5	5	292	239
0.05	0	288	233

[a] Methacholine chloride solution was prepared at 50 mg/ml concentration in deionized water and diluted with PBS pH 7.4.

3. Results and discussion

3.1. Method validation

Method was developed and validated using recommendations defined by ICH guideline for method validation [23].

3.1.1. Specificity

Specificity is the ability of the method to measure the analyte in the presence of all the potential impurities. The specificity with regards to hydrolysis of methacholine products, i.e. acetic acid and β-methylcholine was investigated. The method employed permits a perfect separation between hydrolysis products and methacholine. Under these chromatographic conditions, the retention time of acetic acid and methacholine is 1.9 ± 0.03 and 5.4 ± 0.07 min, respectively (Fig. 1). β-methylcholine was not detected at 210 nm (Fig. 1). In the formulation samples of methacholine chloride, we have verified that potential excipients (sodium chloride or phosphate buffer) did not interfere with the peak of methacholine (data not shown).

3.1.2. Linearity

A linearity study was carried out to determine whether this method can measure accurately different concentrations of methacholine chloride. Five reference solutions containing 80–120% of the concentration of methacholine chloride as used in the assay preparation were tested individually. The response of each individual sample was recorded and the following calculations were performed. The linear equation of the curve obtained by plotting the peak area of methacholine at each level prepared versus the concentration of each sample was calculated using the least square method. The regression equation for methacholine was $y = 1794.33x - 313.37$ and correlation coefficient $(r2) = 0.9990$. Linearity was checked

for 3 consecutive days for the same concentration range from the different stock solutions. The average slope value of methacholine was 1793.90 ± 32.76. Moreover, we have verified with a Student's t-test that intercept was not statistically different from zero. Calculated t value was 0.283 which — with 13 degrees of freedom — is not statistically significant ($t = 2.16$, for $P = 0.05$).

3.1.3. Accuracy

After having verified the linearity and the proportionality of the method as described above, the accuracy of the assay defined as the percentage of the systematic error, is calculated as deviation agreement between the measured value and the true value. Accuracy was evaluated by assaying freshly prepared solutions at 50 mg/ml of methacholine. The accuracy results in terms of percentage recoveries are shown in Table 1. A recovery rate of $99.34 \pm 0.49\%$ was observed. The recovery rate determined confirms the high accuracy of the methacholine assay.

3.1.4. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements of a homogeneous sample. We have studied repeatability also called intra-assay precision and intermediate precision that expresses within-laboratories variations. Repeatability was estimated for four determinations at $100 \pm 5\%$ of the test concentration of the methacholine chloride (50 mg/ml). Intermediate precision of the method was evaluated by assaying freshly prepared solutions of methacholine at 50 mg/ml on three different days with two different analysts. A one-way analysis of variance (ANOVA) was used to evaluate the variance. The results are given in Table 2. A one-tailed F-test is carried out to test whether the mean squares differed significantly. The critical value of F for 2 and 9 degrees of freedom was 4.256 ($P = 0.05$). The test statistic from the table is not greater than the F critical value. Thus, the null hypothesis is correct. There is no statistically significant difference. These data indicate that the assay method is reproducible within the same day and within different days and the precision could be calculated. Precision of the method was expressed as the percentage coefficient of variation (CV) of repeatability and intermediate precision, respectively. The results are shown in Table 3.

3.1.5. Robustness

The robustness of a method is defined by the ability to remain unaffected by small but deliberate variations in the method parameters. In our study, robustness and statistical analysis of the responses were developed using recommendation defined by procedure [24]. The robustness of methacholine assay method was examined by varying the following factors: (A) pH of the mobile phase, (B) percentage acetonitrile in the mobile phase, (C) concentration sodium heptanesulfonate in the aqueous phase. Each factor was examined at two levels (the extreme levels), which were respectively chosen smaller and larger than the operating conditions (nominal levels) (Table 4). These factors were examined in a full factorial design for three factors, thus the number of experiments ($N = l^n$) corresponds to all possible combinations of selected factors (n) and levels (l). In our study, we have tested three factors at two levels, eight experiments are required. Quantitative responses (peak area) measured during the tests are given in Table 5. The effect (coefficient) of each factor on the response (y_i) can be defined by a_i and is given by

$$a_i = \frac{\sum_{j=1}^{N} y_{i+} - \sum_{j=1}^{N} y_{i-}}{N}$$

The significance of this difference is evaluated with a Student's t-test using the standard deviation (S.D.) calculated from the results of experiments (Table 6).

3.1.6. Stability of methacholine chloride solutions

The stability of methacholine chloride solutions in water and phosphate buffer pH 7.4 was studied at different temperatures (30 ± 2, 40 ± 2°C, mean $\pm$ range), and at various times over a 40-day period (under refrigeration 5 ± 3°C). These classic temperatures were chosen because their use is recommended when determining certain drug stability under regulated conditions [24]. The per-

centage of degradation in methacholine chloride solution (50 mg/ml) in water and PBS pH 7.4, at different temperatures is shown in Fig. 2and Fig. 3.

The hydrolysis mechanism of methacholine chloride occurs via two paths well-established [10]. Briefly, methacholine chloride (structure represented in Fig. 3) undergoes base-induced hydrolysis, giving two products of degradation, β-methylcholine (a quaternary ammonium cation) and acetic acid. On the other hand, methacholine is susceptible to produce H^+ through the capacity of its quaternary ammonium cation to generate hydronium ions with water, which consequently lowers pH in solutions. At 4°C, kinetic determination of hydrolysis in methacholine chloride solution (50 mg/ml) shows no hydrolysis in either aqueous solutions and remained unchanged over a 40-day period, but PBS shows a decrease in methacholine chloride concentrations (Fig. 2). When methacholine is dissolved in PBS at 30°C, degradation of 5.5% is observed over 40 days (Fig. 2). Under these conditions, we found that methacholine chloride exhibits maximum stability in water solution rather than PBS, in accordance with observations from previous work [10,11]. Many authors have reported stability studies of methacholine chloride solutions in 0.9% NaCl. But hydrolysis of methacholine in water has not been determined. On the other hand, we have studied the consequences of heat sterilisation (120°C during 20 min) on bulk methacholine chloride solution (50 mg/ml) in water and phosphate buffer pH 7.4. After sterilisation, we observed no sensitive modification of methacholine chloride concentration (data not shown).

4. pH and osmolality of methacholine solutions

Solutions from 0.03 to 25 mg/ml are classically used in inhalation challenges. Hospital pharmacies must prepare methacholine solutions extemporaneously with appropriate diluent before diagnostic test. Osmolality and pH are important to consider when examining mechanisms of bronchoconstriction. Although, different studies have shown that pH and osmolality have a significant role in enhancing the bronchoconstrictor effect [15–18]. Inhalation of acid aerosols has been shown to induce bronchoconstriction in asthmatic subjects [15,16]. A synergetic effect between low pH and hypo-osmolality has been shown [25]. For these reasons, we have controlled pH and osmolality evolution in various concentrations of methacholine.

pH and osmolality measurements of the unbuffered and buffered methacholine solutions are shown in the Tables 7 and 8, respectively. Methacholine chloride solutions prepared from deionized water are strongly hypo-osmolar (< 120 mOsm/kg) at 10 mg/ml and methacholine chloride solutions from 50 to 0.05 mg/ml prepared with deionized water or 0.9% sodium chloride are acidic. The results demonstrated that methacholine solutions prepared with deionized water at 50 mg/ml and diluted with PBS pH 7.4 from 25 to 0.05 mg/ml are a good compromise between pH and osmolarity and could be used in hospital pharmacies.

5. Conclusion

Methacholine chloride rapidly decomposes in such solvents because extensive hydrolysis occurs under such basic conditions. In conclusion, methacholine chloride solutions undergo hydrolysis if the pH exceeds 6. Solutions of higher pH will react faster owing to an increased amount of hydrolysis ions (more basic), and the rates of reaction can vary between different basic buffers of similar pH because their buffering capacities may vary. If methacholine solutions are prepared using basic buffers, rapid deterioration occurs. Our findings clearly demonstrate that dilution conditions of methacholine chloride solutions can have a profound effect on the rate of degradation. These data reveal that temperature and type of solvent influence the degradation rate of solutions of methacholine chloride.

Thus, in accordance with these observations, methacholine chloride must be prepared in sterile water for bulk solution at 50 mg/ml, stored at 4°C to prevent degradation, diluted with PBS pH 7.4 extemporaneously and used in hospital pharmacies for inhalation challenges from 5 to 40 mg/ml.

Moreover, this method was extensively validated and can be used to study stability on methacholine solution.

References

[1] F.E. Hargreave, G. Ryan, N.C. Thompson, P.M. O'Byrne, K. Latimer, E.F. Juniper, J. Dolovich, J. Allergy Clin. Immunol. 68 (1981) 347–355.

[2] S. Spector, T.J. Luparello, M.T. Kopetzky, Am. Rev. Respir. Dis. 113 (1976) 43–50.

[3] J.E. Fish, R.R. Rosenthal, G. Batra, Am. Rev. Respir. Dis. 113 (1976) 579–586.

[4] M.M. Miller, J.E. Fish, R. Patterson, J. Allergy Clin. Immunol. 60 (1977) 116–120.

[5] P.A. Eggleston, J. Allergy Clin. Immunol. 63 (1979) 104–110.

[6] G. Wassmer, R.A. Jorres, J. Heinrich, M. Wjst, P. Reitmeir, H.E. Wichmann, Eur. J. Med. Res. 21 (1997) 47–54.

[7] H. Chai, R.S. Farr, L.A. Froehlich, D.A. Mathison, J.A. McLean, R.R. Rosenthal, A.L. Sheffer, S.L. Spector, R.G. Townley, J. Allergy Clin. Immunol. 56 (4) (1975) 323–327.

[8] B.E. Sekerel, Y. Saraclar, A. Tuncer, G. Adalioglu, F. Cetinkaya, Turk. J. Pediatr. 39 (2) (1997) 165–172.

[9] N.C. MacDonald, C.K. Whitmore, M.C. Makoid, J. Cobby, Am. J. Hosp. Pharm. 38 (6) (1981) 868–871.

[10] B.L. Watson, R.A. Cormier, R.J. Harbeck, Respir. Med. 92 (3) (1998) 588–592.

[11] R.D. Hayes, J.R. Beach, D.M. Rutherford, M.R. Sim, Eur. Respir. J. 11 (4) (1998) 946–948.

[12] M.R. Pratter, T.F. Woodman, R.S. Irwin, B. Johnson, Am. Rev. Respir. Dis. 126 (4) (1982) 717–719.

[13] M. Windholz, The Merck Index, 10th ed., Merck, Rahway, 1983, p. 850.

[14] W.M. Alberts, P.R. Ferguson, J.W. Ramsdell, Am. Rev. Respir. Dis. 127 (3) (1983) 350–351.

[15] J.M. Fine, T. Gordon, D. Sheppard, Am. Rev. Respir. Dis. 136 (5) (1987) 1122–1126.

[16] D.W. Cockcroft, B.A. Berscheid, Thorax 37 (1982) 133–136.

[17] W.L. Eschenbacher, H.A. Boushey, D. Sheppard, Am. Rev. Respir. Dis. 129 (2) (1984) 211–215.

[18] D. Sheppard, N.W. Rizk, H.A. Boushey, R.A. Bethel, Am. Rev. Respir. Dis. 127 (6) (1983) 691–694.

[19] T.F. Woodman, B. Johnson, R.K. Marwaha, J. Liq. Chrom. 5 (1982) 1341–1348.

[20] M.J. van der Schans, J.C. Reijenga, F.M. Everaerts, J. Chrom. A 735 (1996) 387–393.

[21] S. Henn, P. Monfort, J.H. Vigneron, M.A. Hoffman, M. Hoffman, J. Clin. Pharm. Ther. 24 (5) (1999) 365–368.

[22] International Conference on Harmonization, Draft Guideline on Validation Procedures: Definitions and Terminology, March 1, 1995, Federal Register, Vol. 60, pp. 11260.

[23] International Conference on Harmonization, Draft Guideline on Stability Testing of New Drug and Products, November 1999, issued as CPMP/ICH/2736/99.

[24] Guide de Validation Analytique, Rapport d'une commission de la SFSTP, STP Pharm. Prat. 5 (1995) 17–35.

[25] J.R. Balmes, J.M. Fine, D. Christian, T. Gordon, D. Sheppard, Am. Rev. Respir. Dis. 138 (1998) 35–39.