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LC determination of diphemanil methylsulfate: application to stability study of stored pharmaceutical formulations

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Abstract

Diphemanil methylsulfate (DMS) is a synthetic antimuscarinic agent classically used in infants for vagal hypertonia-related symptoms. A normal-phase, isocratic liquid chromatographic method was developed for the quantitative determination of DMS in bulk drugs and in pharmaceutical forms. The method has been completely validated and robustness of this method has been studied. The limit of detection (LOD) for DMS impurities namely, impurity 1 and 2 were found to be 11 and 46 ng/ml. The limit of quantitation (LOQ) was found to be 49 and 139 ng/ml for impurity 1 and 2, respectively. The stability studies have been performed for 2 and 10 mg DMS tablets subjected at various temperatures: 25 °C (long term storage condition) and 40 °C (accelerated storage condition) for 18 and 6 months, respectively. At 25 °C, the samples were found to be stable for the study period. At 40 °C, 2 and 10 mg DMS tablets showed degradation up to 5 and 10% over a 6-month period.

Keywords: Diphemanil methylsulfate; Normal-phase high-performance liquid chromatography; Impurities; Method validation; Stability

1. Introduction

Diphemanil methylsulfate (DMS) is known to be an atropin-like drug, currently used in some infants suffering from vagal bradycardia [1]. The bradycardia due to vagal hyperreflectivity has been suggested to play a role in the pathogenesis of the sudden infant death syndrome [2]. DMS has been used in the past in adults for gastrointestinal disorders and more particularly in the management of peptic ulcer. More recently, DMS was used in the Angelman's syndrome and severe vagal hypertonia [3]. However, some authors have shown that this drug may induce an atrio–ventricular blockage in premature babies [4].

An official methodology was described in the USP XXII using a spectrophotometric procedure for the determination of the concentration of

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DMS in tablets [5]. This method is not specific as assays were carried out with wavelengh detection set at 250 nm. Moreover, this procedure is not easy and requires a solvent extraction. More recently, some authors have developed a gas liquid chromatographic method to study the kinetic parameters of DMS after oral administration [6]. Usually, DMS have been used from 6 to 8 mg/kg per day in premature infants [7]. Actually in France, DMS is not commercialised as a drug yet. However, hospital pharmacies are likely to prepare some pharmaceutical forms with DMS bulk powder. So far to our present knowledge, no LC methods were reported for the analysis of DMS. Thus, it was useful to develop a LC method for the evaluation of its purity and its quantitative determination. In the present work, we have developed and validated a high performance liquid chromatographic (HPLC) method to follow the stability of the DMS, tablets which dosages are 2 and 10 mg at various temperatures: $+25$ and $+40$ °C for 18 and 6 months, respectively.

2. Experimental

2.1. Chemicals

Diphemanil methylsulphate 4-(diphenylmethylene)-1,1-dimethylpiperidinium methyl sulfate (DMS) was obtained from Schering Plough (Hérouville-St-Clair, France) and preserved in its original tight container. This structure of DMS is presented in Fig. 1. Sodium acetate was purchased from Merck (Nogent sur Marne, France). Acetonitrile HPLC grade and acetic acid, were purchased from Carlo Erba (Val de Reuil, France). Water was deionized and filtered through a Milli-Q water purification system from Millipore (Molsheim, France).

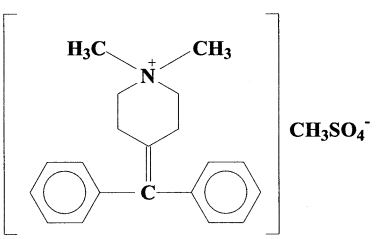

Fig. 1. Chemical structure of DMS.

2.2. Equipment

The liquid chromatographic system consisted of a thermo separation product (TSP) P4000 pump, a TSP AS 3000 auto sampler equiped with a 20 μl loop injector and a TSP UV 2000 detector (Les Ulis, France). The HPLC system was piloted by a PC 1000 software (TSP). Detection wavelength was set at 254 nm. A normal phase column Novapack® CN HP 150 × 3.9 mm with a grain size of 4 μm (Waters S.A Saint-Quentin en Yvelines, France) was used.

2.3. Sample preparation

The independent stock solutions of DMS (0.6, 0.8, 1, 1.2 and 1.4 mg/ml) were prepared by dissolving appropriate amounts of the substance in mobile phase, in 100.0-ml volumetric flasks. DMS solutions were prepared from the stock solutions by taking 10 ml, in 100.0-ml volumetric flasks and made up to the mark with mobile phase. Solution were prepared extemporaneously before being used. In these conditions, we did not notice any degradation of DMS solutions.

2.4. Stability study

Diphemanil tablets 2 and 10 mg have been stored in temperature-controlled: 25 ± 2 °C/60 ± 5% and 40 ± 2 °C/75 ± 5% RH (mean ± range) for 18 and 6 months, respectively. At final time, ten tablets of 2 and 10 mg of DMS were weighed and finely ground using agate mortar and pestle. About 50 and 10 mg of the ground material for 2 and 10 mg DMS tablets, respectively, which is equivalent to 1 mg of the active substance, were introduced in a 100-ml volumetric flask. About 50 ml of mobile phase was shaked by mechanical means for 15 min and mobile phase was added to volume, mixed, and filtered through a 0.45 μm Gelman Acrodisc GHP GF filter. The samples were assayed in duplicate.

2.5. Chromatographic conditions

Separation was carried out isocratically with the following solvent containing acetonitrile, 0.075 M aqueous sodium acetate buffer (pH 4.50) (25/75, v/v). The pH of sodium acetate buffer was adjusted with acetic acid. The mobile phase was filtered under vacuum through a 0.45 μm GHP filter. All separations were performed at room temperature. Under these conditions and with a flow rate of 1 ml/min, the retention time of DMS was 5.09 ± 0.07 min.

3. Results and discussion

3.1. Method validation

Method was developed and validated using recommendations defined by ICH guidelines for method validation [9,10].

3.1.1. Specificity

An analytical method is specific if it guarantees that the measured signal is only related to the substance intended to be analysed (targeted compound) in the presence of all the potential impureties. The specificity with regards to intermediate products of the synthesis was investigated. The method employed enables a perfect separation between both identified intermediate products arising from the route of synthesis, but named for confidential reasons, impurity 1 and 2, respectively. Under these chromatographic conditions, the retention time of impurity 1 and 2 is 2.77 ± 0.005 and 4.12 ± 0.008 min, respectively (Fig. 2). In the formulation samples of DMS, we have checked that potential excipients (microcrystalline cellulose and magnesium stearate) did not interfere with the peak of DMS (Fig. 2).

3.1.2. Linearity

A linearity study was carried out to determine whether this method can measure accurately different concentrations of DMS. Five reference solutions containing 0.6–1.4 mg/ml of DMS were tested individually. The target analyte concentration of DMS was taken as 1 mg/ml. 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 DMS at each level prepared versus the concentration of each sample was calculated using the least square method. The regression equation for DMS was $y = 3594.51x - 1919.11$ and correlation coefficient (r^2) = 0.9990. Linearity has been checked for 3 consecutive days for the same concentration range from the different stock solutions. The average slope value of DMS was 3594.94 ± 40.62. Moreover, we have verified that the intercept was not statistically different from zero with a Student's t-test. Calculated t value equalled 0.574 which—with 13 degrees of freedom—is not statistically significant ($t = 2.16$, $P = 0.05$). An additional analysis was conducted on the statistical residues (Y observed $- Y$ estimated). The results are presented in Fig. 3. It demonstrates that they are distributed randomly around the zero value. This confirms the choice of a linear model.

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 1 mg/ml of DMS. The accuracy results in terms of percentage recoveries are shown in Table 1. A recovery rate of $100.51 \pm 1.70\%$ was observed. The recovery rate determined confirms the high accuracy of the DMS assay.

3.1.4. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample. In this study, we have measured the precision at two levels, repeatability and intermediate precision. Repeatability, also called intra-assay precision, expresses the precision under the same operating conditions over a short interval of time, whereas, intermediate precision expresses within

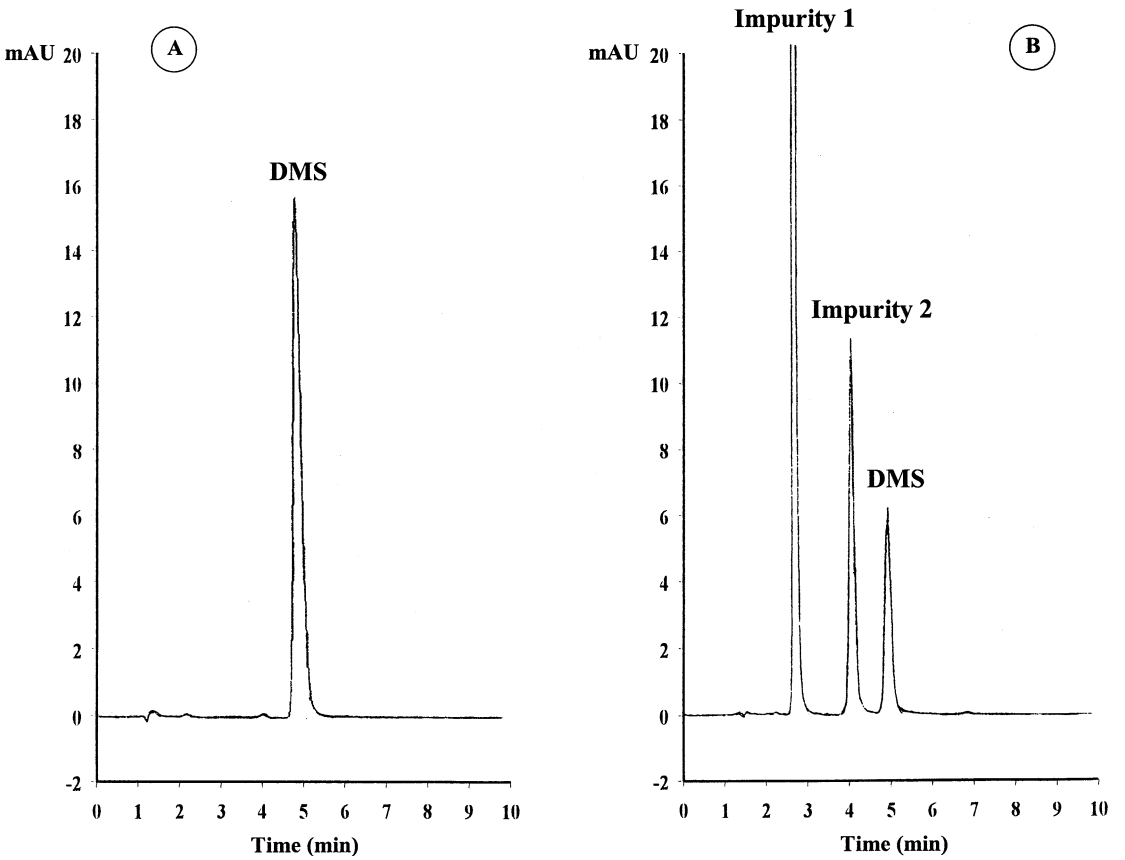

Fig. 2. Chromatograms resulting from the analysis of DMS solution in mobile phase (A), DMS (1.7 μg/ml), impurity 1 (3.3 μg/ml) and impurity 2 (3.2 μg/ml) solution in mobile phase (B). Detection wavelenghth, 254 nm. Chromatographic conditions are as described in Section 2.

laboratory variations. Repeatability was estimated for seven determinations at 1 ± 0.05 mg/ml of DMS. Intermediate precision of the method was evaluated by assaying freshly prepared solutions of DMS in presence of excipients on the 3 different days. 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 significatively. The critical value of F for 2 and 18 degrees of freedom was 3.554 ($P = 0.05$). The statistic test 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 respectively, as the relative standard deviation (R.S.D.) of repeatability and intermediate precision. The results are shown in Table 3.

3.1.5. Limit of detection and limit of quantitation

The limit of detection (LOD) represents the concentration of analyte that would yield a signal-to-noise ratio of 3 in accordance with ICH guidelines [9,10]. LOD for impurity 1 and 2 for 20 μl injection volume were 11 and 46 ng/ml, respectively.

The limit of quantification (LOQ) represents the concentration of analyte that would yield a signal-to-noise ratio of 10 [9,10]. LOQ for impu-

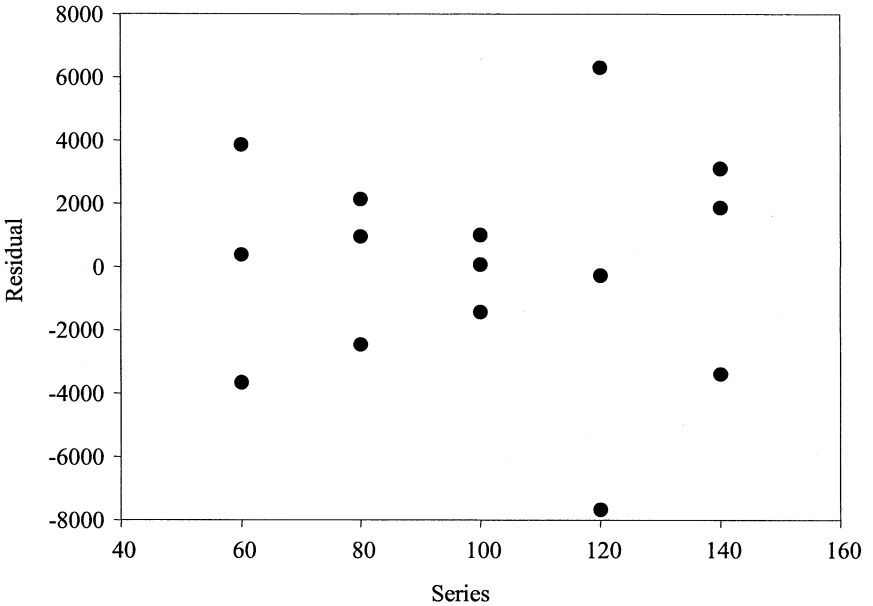

Fig. 3. Scatter plott residuals.

rity 1 and 2 for 20 μl injection volume were 34 and 139 ng/ml, respectively.

3.1.6. 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 recommendations defined by ICH guideline procedure [11]. The robustness of DMS assay method was examined by varying the following factors: (A) percentage of acetonitrile in the mobile phase, (B) pH of the mobile phase, (C) temperature of the column. Each factor was examined at two levels (the extreme levels), which were 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 versus the response (y_i) can be defined by a_i and is given by:

Table 1
Accuracy in the assay determination of DMS

Day of analysis	Taken (g)	Recovery (g) $n = 7$	Recovery (%)
1	0.1019	0.1019	100.04
	0.1002	0.1013	101.15
	0.1001	0.0996	99.56
	0.1007	0.1016	100.91
	1.1040	0.1003	96.41
	0.1003	1.1037	103.46
	0.1004	0.1014	101.03
2	0.1017	0.1030	101.37
	0.1003	0.1013	101.04
	0.0995	0.1016	102.07
	0.1020	0.1024	100.40
	0.1001	0.1046	104.46
	0.1031	0.1004	97.43
	0.1023	0.1016	99.36
3	0.1002	0.1008	100.58
	0.1026	0.1026	99.97
	0.0995	0.0996	100.12
	0.1016	0.1024	100.78
	0.1004	0.1008	100.42
	0.1022	0.1023	100.17
	0.1024	0.1027	100.32

Table 2
One way ANOVA

Source of variation	Degrees of freedom	Sum of squares	Mean suares	*F*-ratio
Between days	2	1.286	0.643	0.204
Within days	18	56.744	3.152	
Total	20	58.029		

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

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

3.2. Stability studies

The stability of 2 and 10 mg per tablets of DMS developed and distributed by Pharmacie Centrale des Hôpitaux de Paris has been evaluated. The DMS tablets were stored in temperature-controlled areas at 25 ± 2 °C/60 ± 5% and at 40 ± 2 °C/75 ± 5% RH (mean $\pm$ range) for 18 and 6 months, respectively. These classic temperatures were chosen, because, their use is recommended when determining certain drug stability under regulated conditions [12]. At 25 °C, the amount of DMS remained unchanged, 100.5 and 99.2% of the target analyte in tablets 2 and 10 mg, respectively. At 40 °C, the amount of DMS obtained was 95.8 and 90.3% of the target analyte in tablets 2 and 10 mg, respectively. In these conditions, two degradation products are noticed and exclusively in accelerated testing. We have not studied sensibility of DMS sample to oxidation or light exposition. In this way, we recommend to preserve DMS tablets in a container closure system at 25 °C to prevent degradation eventually. However, DMS is widely used, after dissolution in water, for neonates and premature infants to treat vagal hypertonia related symptoms. For these reasons, we are developping another and a more convenient pharmaceutical form.

4. Conclusion

An isocratic normal-phase liquid chomatography method has been described for the quantitative determination of DMS. This method is more sensitive and more specific that the spectrophotometric method, which is described in USP XXII and which uses UV detection for DMS assays [5].

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

Day of analysis	Mean of concentration (mg/ml)	S.D.	R.S.D. (%)
Intra-day variability ($n = 7$)			
1	1.0143	0.0130	1.29
2	1.0215	0.0134	1.32
3	1.0160	0.0120	1.18
Inter-day variability ($n = 21$)			
	1.0173	0.0126	1.24

Table 4
The factors examined and their levels

Factor	Levels		
	(−)	(+)	(0)
(A) Acetonitrile (%)	22.5	27.5	25
(B) pH	4.05	4.95	4.5
(C) Temperature of the column (°C)	20	30	25

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	−	−	−	+	+	+	−	213 217
2	+	−	−	−	−	+	+	227 707
3	−	+	−	−	+	−	+	229 971
4	+	+	−	+	−	−	−	221 008
5	−	−	+	+	−	−	+	219 293
6	+	−	+	−	+	−	−	221 396
7	−	+	+	−	−	+	−	225 384
8	+	+	+	+	+	+	+	220 768
Mean								222 343
S.D.								5263.79

Table 6
Student's t-test results

	A	B	C	AB	AC	BC	ABC
Σy_{i-}	221 966	220 403	222 976	226 115	223 348	222 917	220 252
Σy_{i+}	222 720	224 283	221 711	218 572	221 338	221 769	224 435
a_i	376.91	1939.92	−632.71	−3771.68	−1004.99	−573.98	2091.74
t-test $= a_i/(\text{S.D.}/\sqrt{N})$	0.203	1.042	−0.340	−2.027	−0.540	0.308	1.124
Conclusion	NS	NS	NS	NS	NS	NS	NS

$t = 2.36$, with seven degrees of freedom, $P = 0.05$. NS, not significant.

Moreover, this LC method does not require a solvent extraction comparatively to the official procedure. No studies have reported stability, nor degradation products of DMS. The other method described in the literature is gas-exchange chromatography, which is more particularly used for pharmacokinetics studies of DMS [6,8]. This LC method is the first procedure described in the literature for DMS assays and stability studies. The method is also applicable for the evaluation of the purity of DMS. The method was extensively validated and it was found to be robust.

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