OPTIMIZATION AND VALIDATION OF THE MOLIBDENUM BLUE METHOD FOR THE DETERMINATION OF DEXAMETHASONE PHOSPHATE IN INJECTIONS

CÁMARA, M.S.; DE ZAN, M.; MANTOVANI, V.E.; ROBLES, J.C.; GOICOECHEA, H.C.*

Laboratorio de Control de Calidad de Medicamentos, Cátedra de Química Analítica I, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, CC 242, Santa Fe (S3000), Argentina. Fax: 54-342-4575205, e-mail: hgoico@fbcb.unl.edu.ar

Received: April 19, 2002. In final form: May 20, 2002

ABSTRACT

We present the optimization and validation of a very simple and rapid method adapting a wel-known reaction to determinate dexamethasone phosphate in injections. The method is based on the reaction of the phosphate group post calcinations with two reagents: sodium molybdate and ascorbic acid, to form molybdenum blue. The most important variables in the calcination step were selected and the process was optimized applying experimental design techniques and response surface methodology. Figures of merit as limits of determination and quantification, analytical sensibility, intra- and inter-assay precision were calculated by using both artificial and commercial samples. Accuracy was determined by mean of a recovery experiment and comparing results obtained on commercial samples with the USP chromatographic method. The substantial reduction of analysis time achieved with the present method in comparison with HPLC and the good figures of merit, make the former one suitable for control analyses of the injection studied.

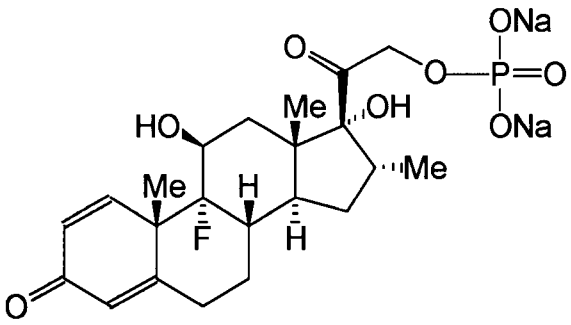
RESUMEN

Se presenta la optimización y validación de un método muy simple y rápido que consiste en la adaptación de una conocida reacción de color para determinar fosfato de dexametasona en inyectables. El método está basado en la reacción del grupo fosfato con dos reactivos de color: molibdato de sodio y ácido ascórbico, para formar azul de molibdeno, luego de realizar una calcinación de la muestra. Se seleccionaron las variables más importantes en la etapa de calcinación y se optimizó el proceso por aplicación de técnicas de diseño experimental y metodología de superficie de respuesta. Se calcularon cifras de mérito tales como límites de determinación y cuantificación, sensibilidad analítica y precisión intra- e inter- ensayo, utilizando muestras artificiales y comerciales. La exactitud se determinó de dos maneras: a) por medio de un experimento de recuperación, y b) comparando los resultados obtenidos sobre muestras comerciales con el método cromatográfico presentado en la farmacopea americana (USP). La considerable reducción del tiempo de análisis necesario cuando se compara este método con el cromatográfico, además de las buenas cifras de mérito obtenidas, lo tornan adecuado para el control de calidad de los inyectables estudiados.

INTRODUCTION

Dexamethasone 21-phosphate [9α-Fluoro-16α-methyl-11β, 17α,21-trihydroxy-1,4-pregnadiene-3,20-dione 21-phosphate] is a synthetic glucocorticoid used for treatment of several pathologies due to its anti-inflammatory and inmunosupressor effects [1]. It yields a symptomatic relief but it has no effects on the development of the underlying disease. Direct intralesional injection of corticosteroid in the treatment of a solitary pulmonary sarcoidosis mass [2] and the efficacy of dexamethasone

for the treatment of antepartum HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome were reported [3].



Dexamethasone 21-phosphaate (disodium salt)

Several chromatographic methods have been reported in the literature to analyse dexamethasone phosphate in different pharmaceutical preparations [4-7] On the other hand, an interesting method using non-volatile buffers in liquid chromatography-mass spectrometry was presented [8]. Other methods as capillary electrophoresis [9] and differential-spectrophotometric determination [10] of dexamethasone sodium phosphate injection were also used. Recently we reported the simultaneous determination of dexamethasone, methylparaben and creatinine in injections by using spectrophotometry and chemometrics tools [11]. Gallego, *et al* [12], also resolved one pharmaceutical mixture containing polymyxin B and trimethoprim with the later technique.

The determination of active compounds content in pharmaceutical preparations is often made by using high-performance liquid chromatography (HPLC). However, this technique has some disadvantages, such as a long time to perform the analysis, and the use of contaminant solvents [13]. The determination of dexamethasone by conventional spectrophotometry without prior separation procedures is not possible due to the overlapping of dexamethasone and excipients UV spectra. Two alternatives are possible: a) the use of multivariate calibration methods [11, 12, 14], and b) application of an indirect spectrophotometric method, by exploiting suitable chromogenic reagents for color development. However, these methods enclose several parameters to be optimized, accordingly experimental design and optimization techniques give us a suitable way to work out the problem. In the present report we present a simple, economical, fast and accurate colorimetric method, based on the reaction of the phosphate group post calcinations with two reagents: sodium molybdate and ascorbic acid, to form moliydenum blue [15, 16]. This method is commonly used to determine phosphorus content in a variety of samples. The test for phosphorus in water, for example, constitutes the ASTM standard D515-88 [17]. The method was adapted to the presently studied pharmaceutical samples, optimized, validated and compared with an official liquid chromatographic method [13].

EXPERIMENTAL

Apparatus

Electronic absorption measurements were carried out on a Perkin-Elmer Lambda 20 spectrophotometer, using 1.00 cm quartz cells. All data were transferred to a PC Pentium 1 GHz microcomputer for subsequent manipulation. Unscrumbler 7.6 CAMO (Trandheim, Norway), Matlab 5.3 (The mathWork Inc.) and Sigma Plot 5.0 (SPSS Inc.) programs were used for processing data (experimental design and statistical tests).

Reagents

All experiments were performed with analytical-reagent grade chemicals. The following reagents were prepared: a) Reagent A: 605 mg of sodium molybdate dihydrate were dissolved in 50.0 ml of distilled water, then 10,7 ml of hydrochloric acid (concentrate) were added and finally the volume was adjusted to 100.0 ml; b) Reagent B: 2.50 g of ascorbic acid were dissolved in 50.0 ml of water and then diluted to 100.0 ml; and c) Reagent C: 3,56 of g arsenic (III) oxide, 1.70 g of sodium hydroxide and 3.00 g of sodium citrate were dissolved in 150.0 ml of water, then the pH was adjusted to 10,7 with sulphuric acid 2 mol l^{-1} and sufficient water was added to produce 200 ml.

Stock solutions of dexamethasone disodium phosphate (1000.00 μg ml^{-1}), were prepared by dissolving the compound in doubly distilled water. A mixture of the usual excipients (matrix) was prepared in distilled water: creatinine (800.00 μg ml^{-1}), propylparaben (300.00 μg ml^{-1}), sodium hydrogen sulphite (2.02 mg ml^{-1}), sodium citrate (1.13 mg ml^{-1}) and sodium hydroxide (1.60 mg mL^{-1}). Magnesium chloride solution 1.5×10^{-3} mol l^{-1} was prepared in distilled water.

Validation sets

Validation samples were prepared by dilution of convenient amounts of stock solutions. The analyte levels in the samples were chosen in order to include a range of 50-150% of the amounts present in the commercial samples as recommended by regulatory agencies [18].

Technique

A volume of 20.0 μl of sample (both standard or commercial samples) was placed in a glace tube and a volume of 80.0 μl of magnesium chloride solution was added. Then the tube was dried in an oven at 100 °C during 20 minutes. The sample was calcined during two minutes on a burner (see below). The colour reaction was carried out in the same tube adding 1.0 ml of Reagent A, 1.0 ml of Reagent B and 1.5 ml of Reagent C, shaking with a vortex shaker and putting the tube in a thermostatic bath at 37 °C during 15 min. The content was quantitatively transferred to a 5.00 ml volumetric flask and water was added up to the mark. Finally, the absorbance was recorded at 702 nm. Magnesium was added as magnesium chloride solution in order to avoid phosphorus volatilisation [19].

Commercial samples

Two commercial samples were tested: Dexamethasone Larjan (Larjan Laboratories, Argentina) and Biocrom (Biocrom laboratories, Argentina). These are injections containing (per ml) 4.0 mg of dexamethasone hydrogen phosphate, and the excipients previously cited.

RESULTS AND DISCUSSION

Experimental design

Experimental design allows a large number of factors to be tested simultaneously and preclude the use of a large number of independent runs when the traditional step-by-step approach is used. Systematic optimization procedures are carried out selecting an objective function, finding the most important factors and investigating the relationship between responses and factors by the so-called response surface methods (RSM) [20]. Usually the optimization criterion is simply an analytical signal, but in this particular case, the elected objective function was a figure of merit: the precision. Thus we minimized the RSD% computed by the analysis of four replicates samples.

Study of significant factors

In order to achieve the significant factors, a Plackett-Burman [20, 21] design was used. The

factors to be studied were: a) Factor A, calcinations time; b) Factor B, amount of magnesium chloride added; c) Factor C, the use or not of volumetric material to obtain the final volume; and d) Factor D, position in which the heating was made in the calcination step. Table 1 shows the coded selected levels for each factor and the response (RSD%) obtained when four replicates were analysed. The application of ANOVA showed that calcination time and amount of magnesium chloride were the most significant factors with an associated probability lower than 0.05 ($p < 0.05$). Therefore these mentioned factors should be investigated in further experiments in order to find out the corresponding optimal values.

TABLE 1. *Analysis of effects whit a Plackett-Burman design.*

Experiment	Factor A[a]	Factor B[b]	Factor C[c]	Factor D[d]	Response: (RSD%)
1	+	+	+	–	0.52
2	–	+	+	+	0.90
3	–	–	+	+	1.30
4	+	–	–	+	0.79
5	–	+	–	–	1.08
6	+	–	+	–	0.77
7	+	+	–	+	0.15
8	–	–	–	–	1.60

[a] Calcinations time: +, 2 min. and –, 1 min.

[b] Amount of magnesium chloride added (ml of 1.5×10^{-3} mol l^{-1} solution): +, 0.050 ml; –, 0.030 ml.

[c] Use or not of volumetric material to obtain the final volume: +, yes; –, no.

[d] Position in which the heating was made in the calcinations step: +, superior; –, inferior.

Optimization of the experimental conditions

A second-order polynomial function was postulated with the aim of obtaining a response map. A full factorial three level design (3^2) was developed. Table 2 shows the coded values and the corresponding response. This design allowed us to obtain the surface response fitting the data to the following polynomial mathematical model:

$$y = \beta_o + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_1 x_2 + \beta_4 x_1^2 + \beta_5 x_2^2 + \varepsilon \qquad [I]$$

were x_i are the analyzed factors (A and B) and β_i are the regression coefficients. As can be seen, equation 1 includes interaction and quadratic terms. After minimum least squares adjustment, the parameters obtained were the following:

$$y = 0.332 + 0.111\, x_1 - 0.213\, x_2 + 0.444\, x_1 x_2 + 0.108\, x_1^2 + 0.425\, x_2^2 \qquad [II]$$

TABLE 2. Full factorial three level designs to apply the surface response methodology.

Experiment	Factor A[a]	Factor B[b]	Response (RSD%)
1	+1	−1	0.68
2	+1	0	1.30
3	+1	+1	1.13
4	0	−1	7.70
5	0	0	1.12
6	0	+1	5.28
7	−1	−1	4.20
8	−1	0	5.40
9	−1	+1	0.70

[a] Calcinations time: +1, 4 min; 0, 3 min and −1, 2 min.

[b] Amount of magnesium chloride added (ml of 1.5×10^{-3} mol l^{-1} solution): +1, 0.100; 0, 0.050 and −1, 0.020.

According to the adjustment performed, and deriving the equation 2, the variable values corresponding to minimum response (RSD% = 0.077), consequently maximum precision, were: calcination time, 2 min (coded level: −1), and amount of magnesium chloride solution, 0.080 ml (coded level: 0.8). Figure 1a shows the response surface performed with the parameters previously calculated and Figure 1b shows the corresponding contour plot in which easily can be seen the optimum value of RSD% selected.

Performance

Linearity

The linearity of the method was evaluated by analysing five standard solutions by triplicate. Thus fifteen samples were prepared. The level concentrations were: 2.0, 3.0, 4.0, 5.0 and 6.0 g l^{-1}. These concentrations corresponded to samples with concentrations from 50 to 150% of the amounts of DEX, which are usually present in the commercial samples. This concentration range was selected following literature guidance [18]. The results of fitting a linear model to describe the relationship between absorbance and concentration of DEX were: Absorbance = 123.8 + 84.7 × Concentration. The r^2 statistic was 0.9982, thus the model explains 99.82% of the variability in absorbance.

According to the approach previously established, the calibration range was 2.0 – 6.0 g l^{-1}. The corresponding detection and quantification limits expressed in concentration unit were: LOD = 0.19 g l^{-1} and LQD = 0.65 g l^{-1} respectively. Another parameter, that may be useful for method comparison, is the analytical sensitivity γ. It may be defined as the quotient [22]:

$$\gamma = b/s_{fit} \qquad [III]$$

where s_{fit} is the standard error of estimate and b is the slope of the least square fitting of the calibration data. The analytical sensitivity allows one to compare analytical methods regardless of the specific technique, equipment, and scale employed; and establishes the minimum concentration difference (γ^{-1}) that is statistically discernible by the method across the dynamic range where it is applicable. In the present work, the value of this figure of merit is: γ^{-1} = 0.065 g l^{-1}, and represents a reasonably good parameter.

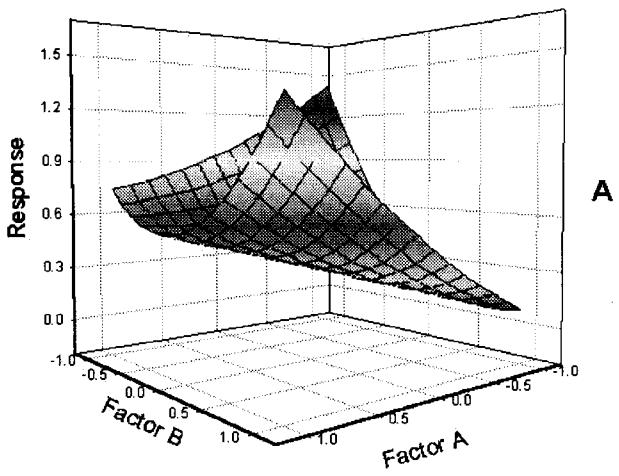


FIGURE 1a. Response surface estimated from the full factorial (3^2) design for calcination time (Factor A) and amount of magnesium chloride solution (B).

Precision

Evaluation of precision estimates, repeatability and intermediate precision, were performed on commercial samples (Biocrom). Ten samples were subjected to analysis during three consecutive weeks and the results are shown in Table 3. As can be seen, the RSD % both intra- and inter-assay are lower than 2 % indicating the very good precision of the proposed methodology. The analysis of variance confirmed that the differences in the mean values among the treatment groups (intermediate precision) are not great enough to exclude the possibility that the differences are due to random sampling variability ($p = 0.401$). On the other hand, no differences were found between the variances obtained in different weeks.

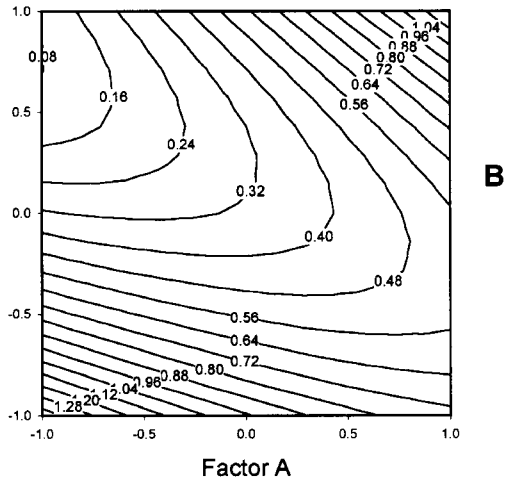


FIGURE 1b. Contour plot corresponding to Figure 1a. The circle shows the variable values corresponding to minimum response (RSD% = 0.077).

TABLE 3. Result s obtained on commercial samples in a precision study during three consecutive weeks.

Samples	Dexamethasone phosphate found (g l^{-1})		
	Week 1	Week 2	Week 3
1	3.72	3.66	3.75
2	3.66	3.68	3.72
3	3.57	3.57	3.57
4	3.68	3.56	3.65
5	3.70	3.73	3.66
6	3.77	3.57	3.74
7	3.55	3.66	3.60
8	3.66	3.67	3.71
9	3.73	3.62	3.70
10	3.64	3.65	3.62
Average	3.67	3.64	3.67
Standard deviation	0.067	0.056	0.062
RSD%	1.88	1.53	1.68
Total average		3.66	
Total standard deviation		0.062	
Total RSD%		1.69	

Accuracy

The accuracy of the method was tested by two ways: a) a recovery experiment and b) a comparison with the USP chromatographic method. In order to carry out the recovery experiment, a matrix sample with the whole excipients was prepared (see experimental) and an amount of DEX corresponding to 100 % of the level in commercial samples was added. Ten replicates were measured and the mean recovery computed was (100.3 ± 0.5) %.

The results obtained when four commercial samples were analysed (three replicates) with both the USP chromatographic method and the present spectrophotometric method are shown in Table 4. As can be seen the recoveries are reasonably good considering the requirements for pharmaceutical quality control assays [18].

TABLE 4. Comparison with the USP chromatographic method. Results obtained when analysing four commercial samples applying both methods.

Samples (different lots)	Spectrophotometric method[a]	Chromatographic method[b]	Recovery[c] (%)
	(g l^{-1})		
1	3.80 (0.05)	3.71 (0.04)	102.4
2	3.78 (0.04)	3.87 (0.04)	97.7
3	3.72 (0.08)	3.69 (0.05)	100.8
4	3.57 (0.07)	3.72 (0.06)	96.0

[a] Three replicates. Values between parentheses correspond to standard deviation.

[b] Three replicates. Values between parentheses correspond to standard deviation.

[c] Recovery is computed having the chromatographic results as reference.

CONCLUSIONS

A very simple, rapid, accurate and precise method to determinate dexamethasone phosphate in injections was optimized and validated. The method is based on the reaction of the phosphate group post calcinations with sodium molybdate and ascorbic acid to form moliydenum blue. Excellent recoveries and coefficients of variations were obtained in all cases. In comparison with conventional HPLC analysis, this method is much faster especially for a large number of assays and is extremely economical. The substantial reduction of analysis time, which is achieved with the present method in comparison with HPLC makes the former one suitable for control analyses of the injection studied.

ACKNOWLEDGEMENTS

Financial support from the Universidad Nacional del Litoral (Projects CAI+D 17-1-41 and 219/ 2002) is gratefully acknowledged.

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