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Method development for betamethasone and dexamethasone by micellar liquid chromatography using cetyl trimethyl ammonium bromide and validation in tablets Application to cocktails

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Abstract

An isocratic liquid chromatographic method for the determination of betamethasone (BM) and dexamethasone (DM) using methylprednisolone (MPL) as internal standard and micellar mobile phases consisting of cetyl trimethyl ammonium bromide (CTAB) and organic modifiers such as propanol, butanol and pentanol has been developed. The effect of organic modifiers, surfactant concentration, temperature and flow-rate on the separation has been studied. Method validation for dexametasone or bethametasone in tablets was carried out using a mobile phase 0.24% pentanol and 32.5 mM CTAB, a flow-rate of 0.5 ml min^{-1}, an Hypersil C_{18} column (60 °C), and UV detection at 243 nm. The recoveries for BM and DM found in the accuracy test were 99 ± 3 and 101 ± 2, respectively. Repeatability and intermediate precision expressed as R.S.D. were lower than 5% for both compounds. The proposed method was applied to cocktails containing both compounds.

Keywords: Betamethasone; Dexamethasone; Pharmaceuticals; Validation; MLC; CTAB

1. Introduction

Betamethasone (BM) and dexamethasone (DM) are epimeric synthetic corticoids (CC) with different configuration of the methyl group on C-16 (structures in Fig. 1), frequently employed as anti-inflammatory, and in the treatment of adrenal cortex insufficiency or allergic diseases [1]. These compounds (prohibited in the EU) have also been used in animal feed [2], fraudulent cocktails (e.g. combination DM–clenbuterol) [3,4], as growth promoters agents in livestock production, and they have been included in the International Olympic Committee doping list [5]. For these reasons, specific and sensitive methods for identification and quantification of these compounds in several samples are required [6,7]. In addition, BM is substituted by DM because it is more expensive than DM and their simultaneous determination is also required.

Micellar liquid chromatography (MLC) using mobile phases containing surfactant concentration above its critical micelle concentration (cmc) is an alternative method to HPLC because of the large number of interactions of solutes with the mobile and stationary phases (enhanced selectivity). In addition, micellar mobile phases are less flammable and expensive, non-toxic and biodegradable. Moreover, the solubilising ability of micelles is one of their most important properties and provides direct injection of untreated samples. The most important drawback of the MLC is the decrease of chromatographic efficiency (poor wetting of the stationary phase and restricted mass transfer) as compared to that obtained in HPLC. To improve chromatographic efficiency in MLC, it has been proposed to

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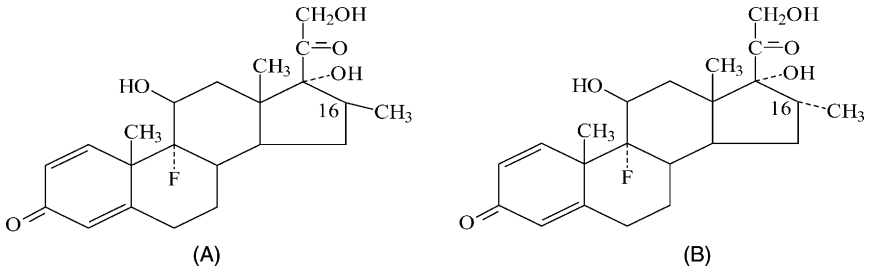

Fig. 1. Chemical structures of betamethasone (A) and dexamethasone (B).

use columns with inner diameter (i.d.) smaller than those employed in HPLC. In addition, the increase of the column temperature and the addition of small amounts of organic modifiers, such as short-chain alcohols, are recommended. The range of concentration of organic modifiers must not be very high, because it might reduce the role of micelles and bring the system closer to a hydroorganic system. Alcohols reduce the loading of the surfactant in the stationary phase (improving the mass transfer and wetting) [8–10].

GC–MS methods for CC have been described [11,12]. However, the analysis is difficult and lacks of specificity for epimeric compounds such as BM and DM [13]. HPLC methods have been employed for CC analysis [14–16] and for the simultaneous determination of DM and BM using UV [17–19] or MS detection [13,20]. Unfortunately, HPLC–MS is not always available in laboratories. MLC was also used for the analysis of single [21] and complex [9] mixtures of CC.

In this paper, a simple, rapid, sensitive, accurate, precise and robust MLC method for the simultaneous determination of DM and BM using methylprednisolone (MPL) as internal standard has been developed. It has been validated (mainly based on the ICH guideline) [22] for Celestone® and Decadran® tablets containing BM or DM, respectively. The proposed method has been applied to cocktail samples containing both DM and BM.

2. Experimental

2.1. Chemicals and reagents

Dexamethasone (9α-fluoro-16α-methyl-11β,17α,21-trihydroxy pregn-1,4-diene-3,20-dione), betamethasone (9α-fluoro-16β-methyl-11β,17α,21-trihydroxypregn-1,4-diene-3,20-dione) and methylprednisolone (6α-methyl-11β,17α, 21-trihydroxy-1,4-pregnadiene-3,20-dione) were purchased from Sigma (St. Louis, MO, USA). Stock solutions of these analytes (1000 μg ml^{-1}) were prepared in methanol. Working solutions (0.2–10 μg ml^{-1}) of a single corticoid or an appropriate mixture of them were also prepared in methanol from stock solutions. *N*-Cetyl-*N*,*N*,*N*-trimethyl-ammonium bromide (CTAB) of analytical-reagent grade were from Merck (Darmstadt, Germany). HPLC-grade methanol (MeOH), 1-propanol (PrOH), 1-butanol (BuOH) and 1-pentanol (PeOH) were purchased from Promochem (Wesel, Germany). Millipore 0.45 μm Nylon filters (Bedford, MA, USA) were also used. Water was purified with a Milli-Q system (Millipore, Molsheim, France). Other used chemicals were of analytical reagent grade.

2.2. Apparatus

The chromatographic system consisted of the following components, all from TSP (FL, USA): a ConstaMetric 4100 solvent delivery system, a SpectroMonitor 5000 photodiode-array detector covering the range 190–360 nm and interfaced to a computer for data acquisition, and a recorder Model CI 4100 data module. A six-port Rheodyne valve with a 20 μl sample loop injector (Cotati, CA, USA), a Jones-Chromatography block heated series 7960 for thermostating columns in the range 30–70 °C (Seagate Technology, Scotts Valley, CA, USA), a vacuum membrane degasser Model Gastor (SAS corporation, Tokyo, Japan) and a bonded silica Hypersil (250 mm × 3.0 mm, i.d. 5 μm) C_{18} column were used. A vortex mixer Mixo-Tub-30 from Crison (Barcelona, Spain) was also used.

2.3. Mobile phases and chromatographic analysis

Isocratic micellar mobile phases were prepared daily mixing well known volumes of PrOH, BuOH, or PeOH, with aqueous solutions of CTAB (prepared with Milli-Q water) by programming the pump (e.g. 0.24% PeOH and 32.5 mM CTAB). Binary mobile phases consisted of PrOH (2–6%) and 20 mM CTAB; BuOH (0.5–2%) and 20 mM CTAB; or PeOH (0.2–0.9) and 20 mM CTAB.

Other mobile phases consisted of 0.5% PeOH and CTAB in the range 15–50 mM; (0.16–0.80%) PrOH and 15–35 mM CTAB. All solvents and mobile phases were firstly filtered under vacuum through 0.45 μm Nylon filters and degassed using a vacuum membrane degasser.

Once the column had been conditioned with the micellar mobile phase (30 min), chromatograms were obtained at the programmed temperature (range 40–70 °C). For optimisation purposes based on the use of different micellar mobile phases, a methanolic solution containing a single compound or an appropriate mixture of them (5 μg ml^{-1}) was injected (20 μl) at a flow-rate of 0.5 ml min^{-1}. Peaks identification and peak purity were performed by comparison of

their retention time and the UV absorbance spectra of the chromatographic peaks with those of reference compounds previously registered by injection of each one individually. In addition, single steroid standards (3 μg ml^{-1}) were spiked to the steroids mixture, and the increase of the corresponding peak area in the chromatogram was checked. Analysis was carried out at 243 nm.

2.4. Sample preparation

Celestone® tablets (Schering Plough) containing 0.5 mg BM per sampling unit (SU) of mean weight = 200 mg, and excipients as starch, dyestuff (FD&C blue no. 1), lactose and magnesium stearate, and Decadran® tablets (Merck Sharp & Dome) containing 0.5 mg DM per sampling unit of mean weight = 100 mg and lactose as excipient, were independently pulverised to obtain a fine powder and homogenised.

2.4.1. Tablets containing BM or DM

Two hundred or 100 mg of the above samples containing BM or DM, respectively, were dissolved in MeOH (10 ml). The methanolic solution was shaken for 5 min, sonicated for 15 min to produce the complete dissolution of the interest compounds and filtered through 0.45 μm nylon filters. Then, 1.0 ml of the above solutions were added with 1 ml 50 μg ml^{-1} MPL (IS) and completed to 10 ml with 20 mM CTAB. The theoretical BM or DM concentration after dilution was 5 μg ml^{-1} (100% BM or DM) and 20 μl were injected into the HPLC system.

Placebo samples were prepared by weighting, mixing and homogenising the excipients of each pharmaceutical and processed in a way similar to the pharmaceuticals.

2.4.2. Cocktails containing BM and DM

Mixtures of 200 mg (Celestone®) and 100 mg (Decadran®), were processed in a way similar to the above samples.

3. Results and discussion

3.1. Column, surfactant and organic modifiers choice

In previous works, the optimisation of the separation of a complex mixture containing CC (including DM and BM) using HPLC and MLC has been studied [16,9,23]. In HPLC, DM and BM were separated using an Hypersil C_{18} column (4.6 mm i.d.), a flow-rate of 1.0 ml min^{-1} and H_2O–THF 72:28 (v/v) as mobile phase [16]. On these grounds, after testing different columns and mobile phases, the separation of a sample containing BM and DM was improved and used to determine these compounds in pharmaceuticals [19]. Higher temperatures and lower flow-rates than those employed in HPLC were used in MLC to improve column efficiency. However, retention times were increased. To keep them constant, smaller i.d.s of the column to operate at similar linear velocities are recommended [8]. In this way, DM and BM were poorly separated using a Hypersil C_{18} column (3.2 mm i.d.), a flow-rate of 0.5 ml min^{-1} and a mobile phase containing sodium dodecyl sulphate (SDS) and typical organic modifiers [9]. However, using CTAB [26] the selectivity improved. On these grounds, to obtain a separation with better performances than those previously obtained for DM and BM, a Hypersil C_{18} column (3.2 mm i.d.; 70 °C), a flow rate of 0.5 ml min^{-1}, 20 mM CTAB (larger than the cmc = 1.3 mM) [24] and PrOH, BuOH and PeOH as organic modifiers, were initially selected.

3.2. Organic modifier optimisation based on separation characteristics

Mobile phases containing 20 mM CTAB and variable composition of short chain alcohols such as PrOH, BuOH and PeOH (the most commonly solvents used in MLC) were optimised (Table 1). For this purpose the separation characteristics of BM and DM in MLC were evaluated. As expected, retention factors, k, for BM and DM decrease as percentage of organic modifier, Φ, increases. In addition, the linear plots of $\ln k$ versus Φ obtained for these compounds indicate that selectivity decreases slightly as Φ increases within the range studied (lines tend to converge). Higher concentrations for these solvents than those indicated in Table 1 provided lower analysis time. However, poor separation characteristics were obtained. Table 1 summarises for each solvent the optimum composition (% OPT) achieved according to resolution between the peaks, R_s, the separation factor, α and analysis time (t_{tot}) involved. From the data in Table 1 and comparing the different organic modifier performances (R_s, α and t_{tot}) 0.5% PeOH was finally selected.

3.3. Effect of CTAB concentration

The effect of CTAB concentration on the separation of BM and DM has been studied at 50 °C in the range 15–50 mM using 0.5% PeOH. As expected, shorter retention factors, k, for DM and BM were obtained as CTAB concentration increased. In addition, the obtained linear plots of k versus CTAB concentration (parallel lines) indicates that selectivity does not significantly change in the range studied; that is,

Table 1
Solvent optimisation for DM and BM separation in MLC using short-chain alcohols

Solvent range	% OPT	k		R_s	α	t_{tot} (min)
		DM	BM			
PrOH, 2–6%	3	13.09	14.11	0.89	1.08	24
BuOH, 0.5–2%	1	15.50	16.75	0.95	1.08	27
PeOH, 0.2–0.9%	0.5	11.94	13.06	0.97	1.09	20

% OPT is the optimum concentration selected for each solvent; k the retention factor; R_s the resolution between DM and BM; and t_{tot} the run time analysis involved. Conditions: 20 mM CTAB, Hypersil C_{18} (150 mm × 3.0 mm i.d., 5 μm; 70 °C) and flow-rate 0.5 ml min^{-1}.

Table 2
Chromatographic data obtained for DM and BM with bivariant optimisation

CTAB (mM)	% PeOH	CTAB/PeOH	k		α	R_s
			DM	BM		
15.0	0.80	18.8	12.70	13.16	1.04	0.95
17.5	0.72	24.3	11.80	12.66	1.07	0.95
20.0	0.64	31.3	11.44	12.32	1.08	0.98
22.5	0.56	40.2	11.08	11.92	1.08	0.96
25.0	0.48	52.1	11.08	12.09	1.09	0.98
27.5	0.40	68.8	11.23	12.18	1.09	0.96
30.0	0.32	93.8	11.75	12.85	1.09	0.96
32.5	0.24	135	12.25	13.43	1.10	0.98
35.0	0.16	219	12.83	14.07	1.10	0.97

CTAB concentration affects the retention factors, k, but not the selectivity. However, resolution decreased in the range 30–50 mM CTAB. A 25 mM CTAB was finally selected as a compromise between resolution and t_{tot}.

3.4. Bivariant optimisation method for the CTAB–PeOH system

A bivariant method using a continuous variation of the concentrations of the CTAB–PeOH system was performed at 70 °C (CTAB concentration was decreased when PeOH was increased). Table 2 summarises the ranges of CTAB and PeOH used, the CTAB/PeOH ratios and the values of k, α and R_s obtained for DM and BM. As can be seen, α and R_s were not modified in a significant way for CTAB/PeOH ratios in the range 31.3–219, affording acceptable separations. These results are not only consistent with those presented above, but also indicate that handling adequate CTAB/PeOH ratios in the range 31.3–219, similar separations can be achieved. In other words, the method presents certain robustness since a slight variation of the CTAB and PeOH concentrations does not change in a significant way the separation performance. A mobile phase 0.24% PeOH and 32.5 mM CTAB allowed the separation of BM and DM practically up to the base line in 18 min, thus being selected for further experiments.

3.5. Effect of temperature

The effect of the temperature on DM and BM retention was studied in the range 40–70 °C using 0.24% PeOH and 32.5 mM CTAB as mobile phase. Table 3 summarises the k-values obtained. A progressive decrease of the retention and selectivity (t_{tot} was always lower than 19 min and α values varied in the range 1.14–1.10) was observed with increasing temperature. However, R_s values (range 0.97–0.99) did not show significant differences. In order to avoid column degradation, higher temperatures are not recommended. From these experiments, 60 °C was selected for the separation of BM and DM. Van't Hoff plots ($\ln k$ versus $1/T$) were constructed with the data of Table 3, showing good linearity ($r > 0.99$). This behaviour evidences that the integrity of the micelle structure is maintained over the temperature range studied. The negative enthalpy values (ΔH) (Table 3), obtained from the slopes indicate that the mass transfer process is exothermic for these compounds and are in agreement with the data reported in the literature [25,26].

Table 3
Retention factors, k, for DM and BM obtained at different temperatures using 32.5 mM CTAB and 0.24% PeOH; R.S.D. < 2%

	DM	BM
40 °C	13.09	14.94
50 °C	12.87	14.50
60 °C	12.61	14.05
70 °C	12.35	13.62
$\Delta H \pm$ R.S.D. (kJ mol^{-1})	-1.67 ± 0.05	-2.50 ± 0.25

In summary, the data obtained from the above studies for these compounds showed to be adequate to develop an analytical method [27].

3.6. Calibration graphs, detection and quantitation limits

Standards containing mixtures of DM, BM and MPL (IS) were prepared at 15 concentration levels in the range 0.2–100.0 μg ml^{-1}, using 5.0 μg ml^{-1} MPL (IS). These solutions were analysed using a mobile phase 32.5 mM CTAB and 0.24% PeOH, a flow-rate of 0.5 ml min^{-1}, an Hypersil column (3.0 mm i.d.; 60 °C) and UV detection at 243 nm. The results were analysed by linear regression. The calibration equations, $Y = A + Bx$ (μg ml^{-1}), were obtained for BM and DM by plotting peak area ratios of BM or DM/IS (Y) versus the concentration (x). The parameters A (intercepts), B (slopes) and r (regression coefficients) were −0.058, 0.259 and 0.999 for DM and −0.034, 0.273 and 0.998 for BM, respectively.

Detection (LODs) and quantitation (LOQs) limits were calculated for a signal/noise (S/N = 3 and 10, respectively) from calibration graphs and the values obtained were 27 and 90 ng ml^{-1} for DM and 20 and 67 ng ml^{-1} for BM, respectively.

4. Analysis of tablets and validation method

4.1. Linearity

Similar calibrations to those performed above were carried out for BM and DM determination in Celestone® and Decadran® tablets. It was performed using placebo samples and seven different amounts of DM and BM in the range 50–150% around the theoretical values (range 2.5–7.5 μg ml^{-1}) and MPL as IS. The calibration equations were consistent with those obtained in Section 3.7. The correlation coefficients, r, found were 0.999 and 0.995 for DM and BM, respectively.

Table 4
Repeatability (RPT), intermediate precision (IP) and accuracy test for samples containing DM and BM

	DM	BM
RPT		
Mean (mg g^{-1})	5.10 ± 0.2	2.46 ± 0.1
R.S.D. (%)	3.6	4.6
IP		
Mean (mg g^{-1})	5.15 ± 0.2	2.42 ± 0.1
R.S.D. (%)	4.0	4.8
R (%)		
90	101 ± 1	100 ± 2
100	102 ± 2	100 ± 1
110	99 ± 2	98 ± 3
Mean	101 ± 2	99 ± 2

4.2. Precision (repeatability and intermediate precision)

The precision was examined by analysing six different tablets ($n = 6$) by only one operator (no. 1), using calibration curves. The repeatability (within run precision) was evaluated by only one operator within 1 day, whereas intermediate precision was evaluated for three different days. The mean and R.S.D. values obtained are shown in Table 4.

4.3. Accuracy

Placebo samples were spiked with different amounts of the active ingredient (DM or BM) at 90, 100 and 110% (in triplicate for each one, $n = 9$) over the theoretical values (2.5 and 5.0 mg g^{-1} for BM and DM, respectively). The mixtures obtained were processed according to the sample preparation method (see Section 2.4) and BM or DM were determined. The mean values of the percent recoveries, *R* (%), are shown in Table 4. As expected, these values are consistent with the theoretical ones for BM and DM.

4.4. Selectivity

Selectivity was assessed by a qualitative comparison of the chromatograms obtained from Celestone® and Decadran® samples and the corresponding placebos. In Fig. 2 are shown the chromatograms obtained from Celestone® and Decadran® samples. Possible interferences due to substances present in tablets were not observed. In addition, a detection and identification process based on retention times and diode array detection (DAD) was carried out [28]. The R.S.D. ($n = 6$) of the retention factors for BM and DM were lower than 1%. The UV spectrum of each peak in the chromatogram was stored and subsequently compared with standards. The spectra were normalised and overlaid. Impurities were investigated further by displaying the spectra obtained at different points across the peak with negative result.

4.5. Robustness

In order to test the robustness of the method, six samples of Decadran® or Celestone® were analysed by two operators (nos. 2 and 3) using standards prepared by themselves and under different chromatographic conditions than those used in the present method (operator no. 1). The working conditions used for the operators and the results obtained are summarised in Table 5.

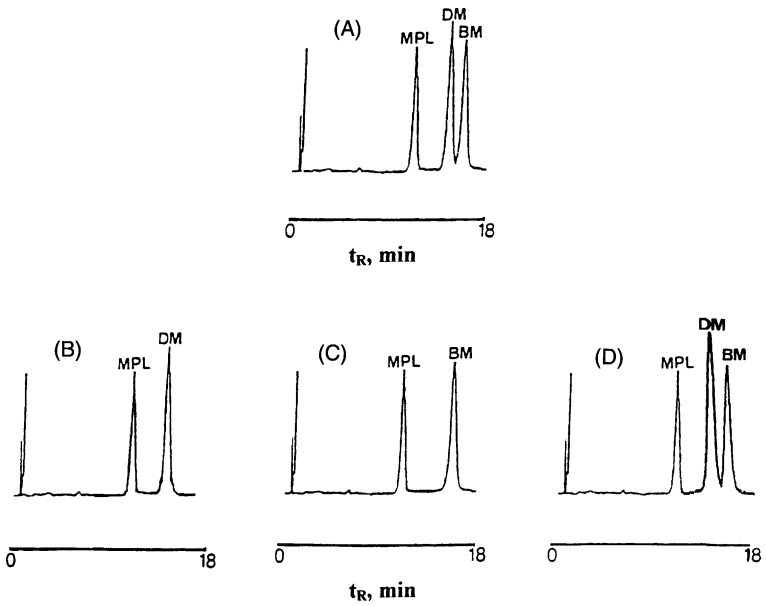

Fig. 2. Chromatograms for BM and DM with UV detection at 243 nm using a mobile phase 32.5 mM CTAB and 0.24% PeOH, a flow-rate of 0.5 ml min^{-1} and an Hypersil column (60 °C); (A) was obtained from a standard mixture of DM and BM (5 μg ml^{-1}), (B) from Decadran® tablets (5 μg ml^{-1} DM), (C) from Celestone® tablets (5 μg ml^{-1} BM) and (D) from a cocktail containing DM and BM (5 μg ml^{-1}). MPL (IS) = 5 μg ml^{-1} in all chromatograms.

Table 5
Chromatographic conditions and results for robustness study

	Operator no. 1	Operator no. 2	Operator no. 3	Mean
Conditions[a]				
F (ml min^{-1})	0.50	0.45	0.55	
λ (nm)	243	242	244	
T (°C)	60	62	58	
Results				
DM				
Mean (mg g^{-1})	5.3	4.9	5.1	5.1
R.S.D. (%)	3.6	2.8	3.0	3.9
BM				
Mean (mg g^{-1})	2.2	2.4	2.5	2.4
R.S.D. (%)	4.4	1.4	5.1	5.3

[a] Hypersil ODS: 150 mm × 3.0 mm; 5 μm. Mobile phase: operator no. 1: 32.5 mM CTAB and 0.24% PeOH; operator no. 2: 30 mM CTAB and 0.28% PeOH; operator no. 3: 34 mM CTAB and 0.20% PeOH.

5. Application to cocktails

The present method was applied to the simultaneous determination of DM and BM in one cocktail ($n = 6$) prepared as indicated in Section 2.4.2. The mean and R.S.D. values were 5.3 mg g^{-1} and 2.6% for DM, and 2.4 mg g^{-1} and 1.2% for BM. Typical chromatograms of DM, BM and their mixtures are shown in Fig. 2. These results were similar to those obtained for the individual determination of BM or DM in tablets.

6. Conclusions

A simple, sensitive, accurate and reproducible and robust MLC method for the epimers BM or DM analysis in tablets using a simple sample preparation procedure has been developed. Moreover, the robustness test indicates that the results are fairly independent on the working conditions since small variations in the main variables of the method do not affect significantly the results. The method achieves the established pharmacopoeias requirements to be used as routine methods for the quality control and stability studies. In addition, the method has been applied to the simultaneous determination of BM and DM in cocktails with similar results to those obtained for the individual determination of BM and DM in tablets. A comparison between the chromatographic results obtained from HPLC [19] and those herein obtained in MLC for DM and BM indicates that with exception to resolution (higher in HPLC versus MLC), selectivity and analysis time are similar for both methods. However, the elution order changes. MLC is cheaper and less toxic and contaminant than HPLC, as a consequence of the flow-rate (0.5 ml min^{-1} in MLC and 1.2 ml min^{-1} in HPLC) and mobile phase composition (24% THF in HPLC and 0.24% PeOH and 32.5 mM CTAB in MLC). In summary, the micellar separation can be considered as an alternative to HPLC.

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