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Short communication

High-performance liquid chromatographic determination of betamethasone and dexamethasone☆

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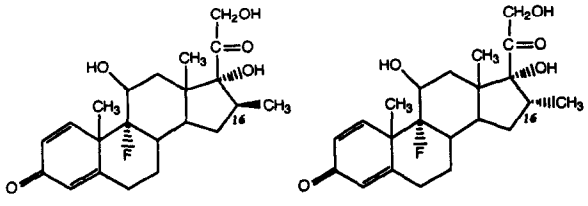
Abstract

A simple method was developed for the determination of glucocorticoid epimers of betamethasone (BTM) and dexamethasone (DXM) by high-performance liquid chromatography with UV detection, using silica as the stationary phase and dichloromethane–ethanol (34:1, v/v) as the mobile phase. The linear range of the method for the determination of BTM and DXM in 1.0 ml of sample solution was over 5–50 nmol. The detection limits (signal-to-noise ratio = 5) of BTM and DXM with an injection volume of 25 μl were 80 and 60 pmol, respectively. The method was satisfactorily applied to the individual determination of BTM and DXM in commercial tablets.

1. Introduction

Betamethasone (9α - fluoro - 16β - methyl - 11β,17α,21 - trihydroxypregna - 1,4 - diene - 3,20 - dione) and dexamethasone (9α - fluoro - 16α - methyl - 11β,17α,21 - trihydroxypregna - 1,4 - diene - 3,20 - dione) (Fig. 1) are potent synthetic glucocorticoids that are widely used for the treatment of inflammation, allergies and other diseases related to glucocorticoid deficiency [1]. The official methods of the United States Pharmacopeia [2] for the individual assay of BTM or DXM in bulk powder and tablet formulations are based on reversed-phase high-performance liquid chromatography (HPLC) with a C_{18} or C_8 column; because of very similar structures of BTM and DXM epimers, no HPLC methods have been reported for the direct separation and determination of BTM and DXM except those of HPLC coupled with analytical derivatization [3,4] and thin-layer chromatography followed by off-line UV absorption spectrophotometry [5] with multiple steps for determination.

In this paper, a simple, specific and rapid HPLC method is described for the determination of BTM and DXM without derivatization. It provides a possible approach for the simultaneous recognition of the epimeric BTM and



Betamethasone **Dexamethasone**

Fig. 1. Structures of betamethasone and dexamethasone.

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DXM, which could be useful in cases of illegal use of DXM as a substitute for BTM, because BTM is more expensive than DXM. The method with the same analytical column was applied to the individual determination of BTM and DXM in bulk samples and formulations.

2. Experimental

2.1. Reagents and chemicals

BTM, DXM, hydrocortisone, 6α-methylprednisolone, prednisolone, prednisone and cortisone (internal standard; I.S.) (Sigma, St. Louis, MO, USA), dichloromethane, chloroform and *n*-butanol (Fisher, Fair Lawn, NJ, USA) and ethanol (Merck, Damstadt, Germany) were used without further purification. All other chemicals were of analytical-reagent grade.

2.2. HPLC conditions

A Waters–Millipore LC system with a U6K injector, a Model 510 pump and a Model 484 UV–Vis detector was used. A Nova-Pak silica (4 μm) column (75 × 3.9 mm I.D.) with a Guard-Pak of Resolve Si column (dead volume 60–75 μl; particle size 5 μm) (Waters–Millipore) and dichloromethane–ethanol (34:1, v/v) as the mobile phase at a flow-rate of 0.7 ml/min were used. The column eluate was monitored at 240 nm. The mobile phase solvents were pretreated with a vacuum filter for degassing.

2.3. Reference standard solution

Approximately 7.85 mg of BTM or DXM was accurately weighed and transferred into a 100-ml volumetric flask with the aid of a suitable amount of the mobile phase, and dissolved in and diluted to volume with the same solvent. The concentration was 200 μ*M*. Appropriate dilutions were made for analytical calibration at various levels.

2.4. Internal standard solution

Approximately 7.22 mg of cortisone was accurately weighed and transferred into a 100-ml volumetric flask with the mobile phase, and dissolved in and diluted to volume with the same solvent. The concentration was 200 μ*M*. Appropriate dilutions were made for analytical calibration.

2.5. Analytical calibration

Five samples each containing 1.0 ml of the reference standard solution over the concentration range 5.0–50.0 μM of BTM or DXM were pipetted in to a series of 10-ml glass-stoppered test-tubes and 0.5 ml of the internal standard solution (40.0 μ*M*) was added and mixed. A 5-μl aliquot of each solution was subjected to HPLC analysis (for the individual determination of the epimer).

Five samples each containing 0.5 ml of BTM and 0.5 ml of DXM reference standard solutions over the concentration range 10.0–100.0 μ*M* were pipetted into a series of 10-ml glass-stoppered test-tubes and 0.5 ml of the internal standard solution (40.0 μ*M*) was added and mixed. A 5-μl aliquot of each solution was subjected to HPLC analysis (for the determination of both epimers).

2.6. Sample preparation

Twenty tablets of BTM or DXM were weighed and finely pulverized. A suitable amount of the resulting powder equivalent to about 0.5 mg of BTM or DXM (synthetic mixtures of BTM and DXM with various proportions of powdered tablet were used for simultaneous determination) was accurately weighed and transferred into a 30-ml test-tube, then 10 ml of water were added for maceration of the powder. After sonication for 15 min, the suspension was transferred into a 125-ml separator and extracted with three 15-ml portions of chloroform–*n*-butanol (95:5, v/v). The combined extracts were filtered through anhydrous sodium sulphate (1 g, moistened with the solvent). The filtrates were collected in a

50-ml volumetric flask and diluted to the volume with the solvent. To 1.0 ml of the resulting solution, 0.5 ml of I.S. solution (40.0 μM) was added and mixed. A 5-μl aliquot of the solution was subjected to HPLC analysis.

3. Results and discussion

Our previous study [3] on the reversed-phase HPLC of BTM and DXM indicated no separation of the epimers, leading to an overlapping single peak. Basically, reversed-phase HPLC is less suitable than normal-phase HPLC (NP-HPLC) for positional isomers. Therefore, simultaneous separation of BTM and DXM by NP-HPLC with a silica column was studied in this work. NP-HPLC of BTM and DXM without derivatization on a silica column with a conventional mobile phase of n-hexane or dichloromethane led to extremely tailing peaks unsuitable for quantification. This can be partially explained by the structures of BTM and DXM in Fig. 1. Each of the epimers has three polar hydroxyl groups in addition to the carbonyl and unsaturated functions that result in a strong interaction between the epimers and the silanol function of the silica. Therefore, an NP-HPLC-compatible mobile phase with an appropriate solvent strength will be essential for the elution of BTM and DXM with good chromatographic properties, and a mobile phase consisting of dichloromethane and ethanol was optimized for the resolution of BTM and DXM.

The response of BTM and DXM was evaluated by measuring the peak-area ratios of the epimer to the I.S. The I.S. was added after the extraction of the epimer in tablet assay simply to keep the I.S. as a relatively constant factor. It would better be added before extraction if evidence for its constant recovery can be proved.

3.1. Mobile phase for NP-HPLC

In the HPLC of BTM and DXM on a silica (4 μm) column (75 × 3.9 mm I.D.), the mobile phase used for the elution of the epimers was studied including various ratios of dichloromethane to ethanol over the range 26:1 to 34:1 (v/v) in order of decreasing elution strength. The results found that the baseline resolution of BTM and DXM can be achieved using dichloromethane–ethanol (34:1, v/v), leading to capacity factors of 4.99 and 3.87, respectively, and a resolution of 1.7.

A typical chromatogram of BTM and DXM is illustrated in Fig. 2, indicating good chromatographic properties of symmetrical peaks and fast separation in less than 7 min. The greater retention of the epimers of BTM in the NP-HPLC system is probably due to the β-orientation of the methyl group at C_{16} that hinders less the α-hydroxyl group at C_{17} from forming hydrogen bonds with the silanol function of the silica stationary phase. The α-orientations of both the methyl group at C_{16} and the hydroxyl group at C_{17} in DXM result in unfavourable hydrogen bonding between the solute and the stationary

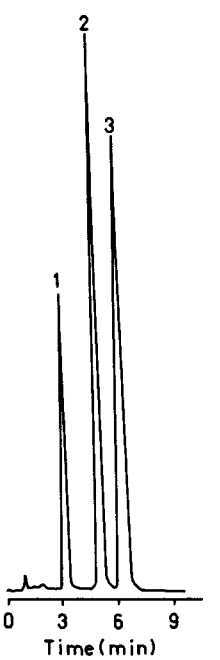

Fig. 2. HPLC of DXM (33 μM)–BTM (33 μM) mixture. Peaks: 1 = cortisone (I.S.); 2 = DXM; 3 = BTM. Conditions: Nova-Pak silica (4 μm) column (75 × 3.9 mm I.D.); mobile phase, dichloromethane–ethanol (34:1, v/v); flow-rate, 0.7 ml/min; UV detection at 240 nm; sample size, 5 μl.

phase, leading to less adsorption and faster elution.

3.2. Analytical calibration

To evaluate the quantitative applicability of the method, five different amounts of BTM and DXM in the range 5–50 nmol were analysed and the linearity between the peak-area ratios (y) and the epimer mass (x, nmol) was examined. The linear regression equations obtained for individual determination ($n = 5$) were $y = (0.05650 \pm 0.00033)x - (0.02797 \pm 0.00443)$ ($r = 0.999$) and $y = (0.05826 \pm 0.00084)x - (0.01801 \pm 0.01010)$ ($r = 0.999$) for BTM and DXM, respectively. The detection limits (signal-to-noise ratio = 5) of BTM and DXM per injection (25 μl) were 80 ± 20 and 60 ± 20 pmol, respectively.

In parallel, analytical calibration for both BTM and DXM based on five different amounts of the epimers each over the range 5–50 nmol resulted in the linear regression equations $y = (0.05678 \pm 0.00096)x - (0.02999 \pm 0.01796)$ ($r = 0.9999$) and $y = (0.05811 \pm 0.00113)x - (0.01329 \pm 0.01721)$ ($r = 0.9999$) for BTM and DXM, respectively. The results for the calibration of the epimers indicated good linearity of the method for the amounts of the epimers *versus* peak-area ratio studied. Because standard BTM and DXM were eluted with different retention times, as shown in Fig. 2, the method can identify which is being analysed when DXM is used as a partial or total substitute for the more expensive BTM in formulations for economic purposes. Demonstration of the application of the method to the analysis of synthetic mixtures of BTM and DXM was shown in Table 1.

3.3. Reproducibility and selectivity

The precision (relative standard deviation, R.S.D.) of the method based on the peak-area ratios for replicate determinations of BTM and DXM each at 40- and 8-nmol levels was studied. The results indicated that the intra-day precisions ($n = 9$) for BTM and DXM at 40 nmol were <0.8% and those at 8 nmol were <2.4%; the inter-day precisions ($n = 9$) for BTM and DXM were <1.0% at 40 nmol and <3.4% at 8 nmol.

The selectivity of the method was examined by spiking samples of BTM and DXM with other glucocorticoids including cortisone, hydrocortisone, prednisone, prednisolone and 6α-methylprednisolone. The results in Fig. 3 indicate that BTM and DXM can be well resolved from other glucocorticoids of oral use, revealing the favourable specificity of the method and its potential use for the direct HPLC of other glucocorticoids after suitable modification.

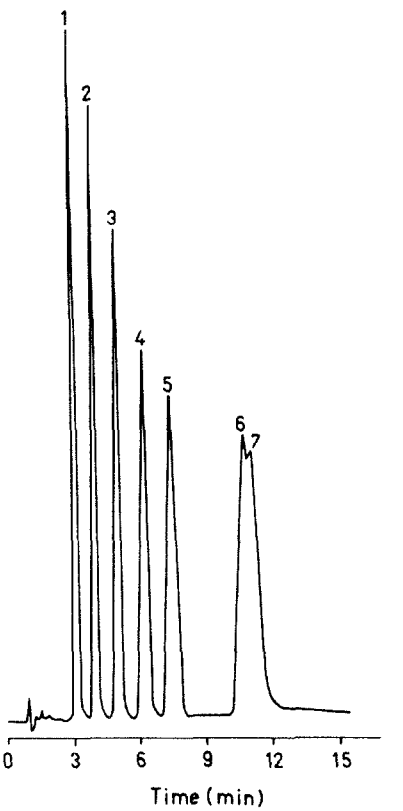

Fig. 3. HPLC of a mixture of standard glucocorticoids. Peaks: 1 = cortisone; 2 = prednisone; 3 = DXM; 4 = BTM; 5 = hydrocortisone; 6 = 6α-methylprednisolone; 7 = prednisone (each at 25 μM). HPLC conditions as in Fig. 2.

3.4. Application

Prior to application of the method to the determination of BTM or DXM in tablets, the solvent system for the extraction of BTM or DXM from tablet was investigated. A finely pulverized tablet of mass equivalent to about 0.5 mg of BTM or DXM was transferred into a 30-ml test-tube and macerated with 10 ml of water and further treated by sonication as indicated in Section 2.6. The resulting suspension was extracted with various proportions (0–15%, v/v) of *n*-butanol in chloroform (3 × 15 ml), leading to the selection of *n*-butanol–chloroform (5:95, v/v) as the optimum. The extraction of BTM or DXM from the water-macerated tablet suspension is facilitated by using water-immiscible chloroform with a suitable amount of *n*-butanol (≥5%, v/v). Because the common excipients such as lactose and starch used for tablet formulations are also polar, containing hydroxyl groups, these result in interaction of the excipient with DXM and BTM and an extraction solvent system with a hydroxyl function is therefore useful for BTM and DXM. This is in accordance with the behaviour of the polar epimers interacting with the silica stationary phase and needs a mobile phase containing ethanol for better elution in NP-HPLC.

The extraction of a known amount of BTM or DXM added to its powdered tablet with *n*-butanol–chloroform (5:95, v/v) resulted in satisfactory recoveries, *e.g.*, 98.7 ± 2.1 and 99.3 ± 1.8% ($n = 3$) for BTM at spiking levels of 0.1 and 0.3 mg, respectively, and 97.7 ± 1.5 and 99.4 ± 1.0% ($n = 3$) for DXM at spiking levels of 0.1 and 0.3 mg, respectively. This reveals the good extraction of BTM and DXM from their tablet diluents with chloroform containing a suitable amount of *n*-butanol. The recoveries were calculated as follows: recovery (%) = (total amount of BTM or DXM found after adding the epimer to its powdered tablet − amount of the epimer found in its powdered tablet before spiking) × 100/amount of the epimer added. The detailed extraction protocol for BTM and DXM is given in Section 2.6.

The method was applied to the determination of BTM and DXM in tablets and synthetic mixtures. The results are given in Table 1; all the analytical values for BTM and DXM tablets fell within the labelled range of 90–110% required by the USP.

In conclusion, an adsorption HPLC method has been developed for the determination of BTM and DXM. The method uses no derivatization reagent in the determination of the epimers; as a consequence, no additional and time-con-

Table 1
Assay results (mean ± S.D.; $n = 3$) for BTM and DXM tablets and their synthetic mixture

Sample	Percentage of claimed content[a]	
	BTM	DXM
BTM tablet		
B_1	96.2 ± 1.7	
B_2	94.0 ± 0.2	
B_3	94.6 ± 1.2	
B_4	91.2 ± 1.6	
B_5	94.6 ± 0.8	
Mean	94.1	
S.D.	1.8	
DXM tablet		
D_1		100.6 ± 1.0
D_2		99.8 ± 1.1
D_3		100.0 ± 1.5
D_4		101.8 ± 1.4
D_5		103.4 ± 0.7
Mean		101.1
S.D.		1.5
Test mixture[b]		
M_1	94.8 ± 1.2	104.0 ± 1.0
M_2	95.1 ± 1.3	100.9 ± 0.6
M_3	93.4 ± 1.0	100.7 ± 0.6
Mean	94.4	101.9
S.D.	0.9	1.8

[a] The labelled amount of BTM and DXM in each tablet is 0.5 mg.

[b] Synthetic mixtures were formulated with various proportions of pulverized tablets of BTM and DXM in mg of labelled amount: M_1 (0.40 mg BTM + 0.10 mg DXM), M_2 (0.25 mg BTM + 0.25 mg DXM) and M_3 (0.10 mg BTM + 0.40 mg DXM).

suming step for analytical derivatization is required. Hence this direct HPLC method is simple, rapid and selective for the determination of BTM and DXM. It seems to be practical and economical for the quality control of BTM and DXM in bulk and pharmaceutical products.

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