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Stereochemical analysis of betamethasone and dexamethasone by derivatization and high-performance liquid chromatography

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ABSTRACT

A simple and economical high-performance liquid chromatographic method has been developed for the simultaneous determination of betamethasone and dexamethasone. The method is based on the derivatization of the structural epimers of betamethasone and dexamethasone with a homochiral reagent, N-carbobenzoxy-L-phenylalanine. The derivatives obtained were easily recognized by a non-chiral silica column with *n*-hexane–dichloromethane–isopropanol (100:100:4, v/v/v) as a mobile phase and a good separation was obtained for quantitation. The method was satisfactorily applied to the determination of betamethasone and dexamethasone in tablets.

INTRODUCTION

Betamethasone (BTM) and dexamethasone (DXM), illustrated in Fig. 1, are highly potent fluorinated glucocorticoids and are widely used for the treatment of inflammation, allergies and adrenal cortex insufficiency. BTM has been reported to have a slightly stronger glucocorticoid effect [1] and is more expensive than DXM.

The official methods of the United States Pharmacopeia (USP) [2] for the determination of BTM and DXM are based on reversed-phase high-performance liquid chromatography (HPLC) with C_{18} and C_8 columns, respectively. Although a number of liquid chromatographic methods [3–13] have been described for the determination of BTM, DXM, or their analogues in various samples, to the authors' knowledge, no HPLC methods have been reported for the simultaneous determination of BTM and DXM. This is mainly due to the close structural similarity of the epimers of BTM and DXM which unfavourably affects their separation.

An attempt was made to increase the stereochemical differences between the epimers by derivatization with a commercially available homochiral reagent, N-carbobenzoxy-L-phenylalanine (N-CBZ-Phe). A good separation of the derivatives of BTM and DXM was given on an achiral silica column.

In this paper, an HPLC–UV derivatization method for the simultaneous determination of BTM and DXM is described. Its application to the determination of BTM and DXM in commercial tablets was satisfactory. Further development of the method for the simultaneous determination of BTM, DXM and their analogues in drugs used illegally, such as for promoting zootechnical performance and enhancing the effect of herbal medicine, will be useful.

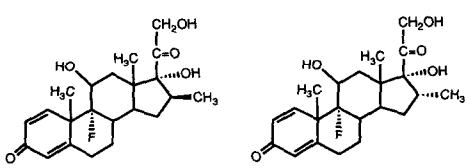

Fig. 1. Structures of betamethasone and dexamethasone.

EXPERIMENTAL

Materials

BTM, DXM and phenacetin (internal standard, I.S.) (Sigma, St. Louis, MO, USA), N-CBZ-Phe, N,N′-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) (TCI, Tokyo, Japan), dichloromethane and *n*-hexane (Fisher, Fair Lawn, USA), and isopropanol and silica gel 60 (70–230 mesh) (E. Merck, Darmstadt, Germany) were used without further treatment and all reagents used were of analytical-reagent grade.

HPLC conditions

A Water-Millipore LC system with a U6K injector and a Model 484 UV–VIS detector was used. A Nova-Pak silica column (75 × 3.9 mm I.D., 4 μm) with a disposable Resolve silica precolumn (10 μm; bed volume < 100 μl) (Waters-Millipore) and a mixed solvent of *n*-hexane–dichloromethane–isopropanol (100:100:4, v/v/v) at a flow-rate of 1.0 ml/min were used. The column eluate was monitored at 240 nm. The solvents were degassed with a vacuum filter before use.

Mass and NMR spectrometry

Mass spectra were obtained on a JEOL JMS-HX 110 mass spectrometer with fast atom bombardment (FAB) of xenon as the ionization mode and an acceleration energy of 10 kV. ^{13}C NMR spectra were recorded in C^2HCl_3 on a Varian VXR 300 spectrometer. The nature of the ^{13}C resonance was deduced from distortionless enhancement by polarization transfer experiments performed by using polarization transfer pulses of 90 and 135°, respectively.

Derivatization procedures

Solutions of BTM, DXM, phenacetin, N-CBZ-Phe and DCC were prepared by dissolving the appropriate amount of the respective compounds in dichloromethane.

Procedure for individual determination. A 0.5-ml volume of BTM or DXM solution was added to a 10-ml glass-stoppered test-tube containing 0.2 ml of phenacetin (I.S.) solution (1.2 m*M*) and 0.1 ml of DMAP solution (0.5 m*M*). A 0.1-ml volume of solution of N-CBZ-Phe (90 m*M* for BTM or 20 m*M* for DXM) and DCC (90 m*M* for BTM or 20 m*M* for DXM) was then added. The reaction mixture was shaken mechanically at 30°C for 30 min. A 10-μl aliquot of the solution was determined by HPLC with UV detection.

Procedure for simultaneous determination. A 0.5-ml volume of a solution of BTM and DXM was added to a 10-ml glass-stoppered test-tube containing 0.2 ml of phenacetin (I.S.) solution (1.2 m*M*) and 0.1 ml of DMAP solution (0.5 m*M*). A 0.1-ml volume of each N-CBZ-Phe (100 m*M*) and DCC (100 m*M*) was then added. The reaction mixture was shaken mechanically at 30°C for 1.0 h. A 10-μl aliquot of the solution was then separated by HPLC.

RESULTS AND DISCUSSION

Direct chromatographic separation of BTM and DXM with reversed- and normal-phase HPLC using an achiral column was briefly studied. Reversed-phase HPLC for the separation of BTM and DXM with a C_{18} column (150 × 3.9 mm I.D.; 4 μm) using common mobile phases (at a flow-rate of 1 ml/min), including acetonitrile–water (40:60, v/v) and methanol–water (40:60, v/v) was performed, but BTM and DXM were eluted as a single peak. The separation of BTM and DXM by normal-phase HPLC using a silica column (75 × 3.9 mm I.D.; 4 μm) with a variety of mixed solvents at a flow-rate of 0.8 ml/min was also studied, including dichloromethane–isopropanol (100:4, v/v) and *n*-hexane–dichloromethane–isopropanol (100:100:8, v/v/v). These all resulted in tailing and incomplete resolution of BTM and DXM. Decreasing the polarity of the mobile phase *n*-hexane–dichloromethane–isopropanol (100:100:6, v/v/v) led to tailing peaks not suitable for chromatographic analysis, as shown in Fig. 2.

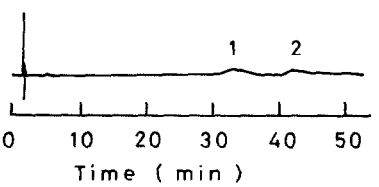

Fig. 2. HPLC chromatogram of an underivatized mixture of BTM and DXM. Peaks: 1 = DXM; 2 = BTM. Conditions: Nova-Pak silica column (75 × 3.9 mm I.D.; 4 μm); mobile phase, *n*-hexane–dichloromethane–isopropanol (100:100:6, v/v/v); flow-rate, 0.8 ml/min; UV detection, 240 nm.

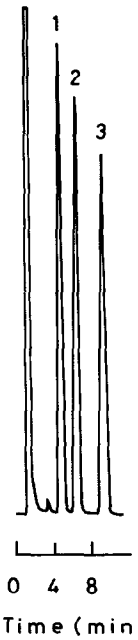

Fig. 3. HPLC chromatogram of the simultaneous determination of a BTM and DXM mixture. Peaks: 1 = Derivative of DXM; 2 = derivative of BTM; 3 = phenacetin (I.S.). Conditions: Nova-Pak silica column (75 × 3.9 mm I.D.; 4 μm); mobile phase, *n*-hexane–dichloromethane–isopropanol (100:100:4, v/v/v); flow-rate, 1.0 ml/min; UV detection, 240 nm.

An analytical strategy for better molecular recognition of BTM and DXM focused on the derivatization of BTM and DXM with a commercially available homochiral reagent, N-CBZ-Phe. As shown in Fig. 3, the resulting derivatives were well resolved by adsorption HPLC with good chromatographic properties of resolution (R_s = 1.59) and selectivity (α = 1.50), indicating that the derivatives of BTM and DXM interact differently with the silica column and the mobile phase. For the optimization of the derivatization conditions for BTM and DXM, some parameters affecting the derivatization reaction were investigated and evaluated by the peak-area ratios of the respective derivative to the I.S. instead of evaluation by the true yield of the derivatives.

Effect of amount of N-CBZ-Phe and DCC

The effects of variable amounts of N-CBZ-Phe and DCC with 0.05 μmol of DMAP on the derivatization of BTM (0.05 μmol) and DXM (0.05 μmol) at 30°C were studied. The results indicated that the amounts of N-CBZ-Phe required for the equilibrium derivatization of BTM and DXM were 9.0 and 2.0 μmol, respectively. The amounts of DCC required for the derivatization of BTM and DXM were also 9.0 and 2.0 μmol, respectively. BTM needs more N-CBZ-Phe to reach equilibrium derivatization in 0.5 h; the derivatization of BTM with smaller amounts of N-CBZ-Phe (2.0 μmol) and DCC (2.0 μmol) resulted in a longer time (about 4.0 h) to attain plateau formation of the derivative with a peak to area ratio equivalent to that obtained with the larger amount. The lower reactivity of BTM is probably because the β-orientation of the two methyl groups at C_{16} and C_{13} sterically affect the esterification of the hydroxyl group at C_{21} with N-CBZ-Phe.

Effect of reaction time

The effects of reaction time were examined for the derivatization of BTM and DXM under the optimum conditions. The results were presented in Fig. 4. The derivatization reaction proceeded under mild conditions and reached equilibrium in a favourably short time of about 0.5 h.

Structural identification of the derivatives

The structural analysis of the isolated derivatives of BTM and DXM was performed by FAB mass spectrometry with 3-nitrobenzyl alcohol as a matrix. The mass spectra obtained are shown in Fig. 5. Quasi-molecular ions of the derivatives of BTM and DXM were found at m/z = 674 $(MH)^+$. This

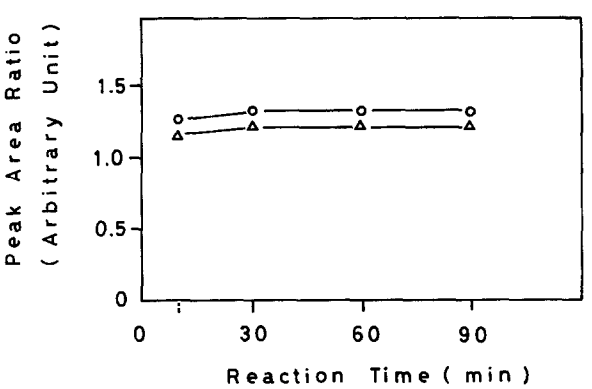

Fig. 4. Effect of reaction times on the formation of the BTM or DXM derivatives. △ = BTM; ○ = DXM.

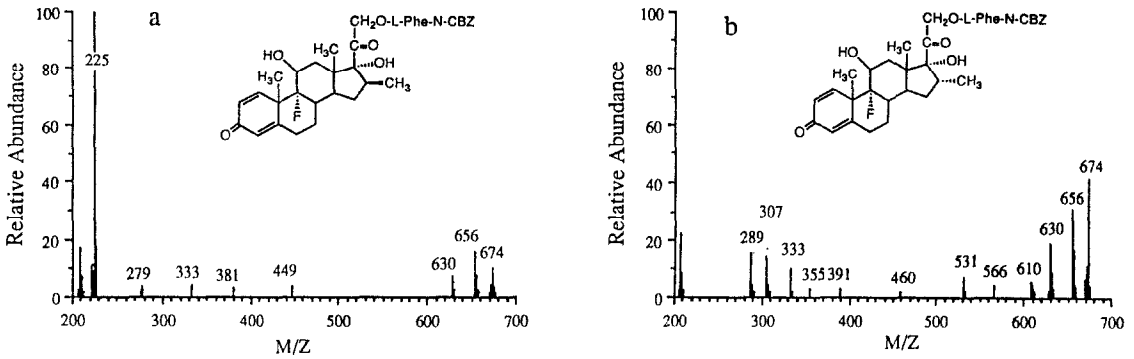

Fig. 5. Mass spectra of (a) BTM and (b) DXM derivatives with FAB ionization. See text for conditions.

indicates that one of the alcohol groups of the epimer was acylated as no higher mass fragments of BTM or DXM were found. In addition, the ion fragments at *m/z* 656 and 333, which correspond to $(MH)^+$ minus water and $(MH)^+$ minus a carbonyl side-chaine at C_{17} on BTM or DXM; the *m/z* fragments at 674 and 333 suggest that the formation of a single ester is from the primary hydroxyl group at C_{21} on BTM or DXM. Furthermore, by comparing the ^{13}C NMR spectra of the derivatives of BTM and DXM with those of underivatized BTM and DXM, an upfield shift of about 7.8 ppm from 212.9 ppm (chemical shift) of C_{20} on BTM to 205.1 ppm of C_{20} on the derivative of BTM and also a parallel upfield shift of 7.4 ppm from 211.7 ppm of C_{20} on DXM to 204.3 ppm of C_{20} on the derivative of DXM were observed. These findings suggest that the hydroxyl group at C_{21} is acylated. The possible hydrogen bonding between the carbonyl group of C_{20} and the hydroxyl group at C_{21} disappears owing to the esterfication of the OH group at C_{21} on BTM or DXM and this leads to the upfield shift of the carbonyl carbon of C_{20} as proposed by Duddeck *et al.* [14]. From the mass and ^{13}C-NMR analyses of the derivatives of BTM and DXM, the position of the ester formation is assigned as at the primary alcohol group of the epimers.

Analytical calibration

Based on the optimum derivatization conditions, the derivatization procedures for the individual and simultaneous determination of BTM and DXM were formulated as described under Experimental. To evaluate the quantitative applicability of this method, five different concentrations of BTM or DXM over the range 1.85–50 nmol (each in 0.5 ml of dichloromethane) were determined to construct a calibration graph. The results indicate good linearity for the determination of BTM and DXM over the range used; for individual determination, the linear regression equations obtained were $y = 0.02539x - 0.002517$ with $r = 0.999$ for BTM and $y = 0.02933x + 0.002376$ with $r = 0.999$ for DXM, where y is the peak area ratio of the glucocorticoid derivative to the I.S., x is the amount in nmol of BTM or DXM and r is the correlation coefficient. Possible linearity to higher concentrations of BTM and DXM was not studied because such concentrations are of no practical use. The detection limits (signal-to-noise ratio 5) of BTM and DXM with a 20-μl aliquot injected were 4.2 and 2.1 pmol, respectively.

Satisfactory results were also obtained for the simultaneous determination of BTM and DXM at five different concentrations in the range 1.85–50 nmol. The linear regression equations obtained were $y = 0.02473x + 0.002410$ with $r = 0.999$ for BTM and $y = 0.02798x + 0.002146$ with $r = 0.999$ for DXM. The reproducibility (relative standard deviation) of the proposed method from ten replicate measurements of 50 nmol of the epimer was 1.43% for BTM and 1.21% for DXM.

Application

One tablet of commercial BTM or DXM was placed in a 10-ml test-tube containing 5.0 ml of distilled water for maceration. It was then triturated with a glass rod and sonicated for 20 min to fully

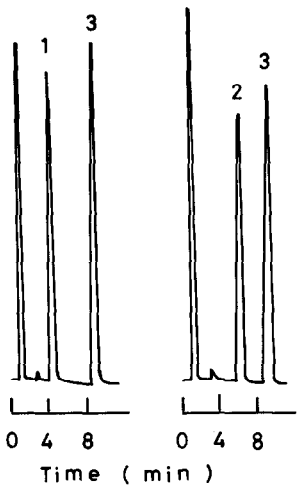

Fig. 6. HPLC chromatograms for the determination of BTM and DXM in tablets. Peaks: 1 Derivative of DXM; 2 = derivative of BTM; 3 = phenacetin (I.S.). Conditions: Nova-Pak silica column (75 × 3.9 mm I.D.; 4 μm); mobile phase, *n*-hexane–dichloromethane–isopropanol (100:100:4, v/v/v); flow-rate, 1.0 ml/min; UV detection, 240 nm.

disintegrate the matrix. The contents of the test-tube were transferred into a 50-ml separator with the aid of 9 ml of dichloromethane and were extracted with an additional three 5-ml portions of dichloromethane. The dichloromethane extracts were filtered through a dichloromethane-treated filter paper into a 25-ml calibrated flask. Dichloromethane was added to the solution and well mixed. A 0.5-ml aliquot of this solution was pipetted into a 10-ml glass-stoppered test-tube and derivatized as described earlier. The HPLC chromatograms obtained are shown in Fig. 6 and the analytical results were presented in Table I. The concentrations of BTM and DXM in the tablets tested were all within 90–110% of the labelled amount of BTM and DXM specified by the current USP. A synthetic test mixture was formulated by mixing an amount of powdered tablets from BTM and DXM equivalent to 0.25 mg of BTM plus 0.25 mg of DXM and this was analysed as described. The results are also shown in Table I. This simultaneous determination of BTM and DXM in a test mixture provides a method to check the illegal use of DXM for BTM or the use of a mixture of DXM and BTM for BTM to reduce costs. The proposed method could therefore be used for the quality control of BTM and DXM in pharmaceutical products.

TABLE I

ASSAY RESULTS FOR BTM AND DXM TABLETS AND THEIR TEST MIXTURE OBTAINED FOR TWO COMMERCIAL SOURCES

Results given as mean ± S.D.

Sample	Amount found (mg per tablet)	Percentage of manufacturer's claim (%)
BTM (0.5 mg per tablet)[a]		
B_1	0.457 ± 0.003	91.4
B_2	0.462 ± 0.004	92.6
B_3	0.468 ± 0.003	93.6
B_4	0.455 ± 0.002	91.0
B_5	0.475 ± 0.005	95.0
B_6	0.471 ± 0.004	94.2
	Mean recovery (%)	93.0
	S.D. (%)	1.58
DXM (0.5 mg per tablet)[a]		
D_1	0.486 ± 0.005	97.2
D_2	0.492 ± 0.004	98.4
D_3	0.487 ± 0.005	97.4
D_4	0.488 ± 0.004	97.6
D_5	0.507 ± 0.006	101.4
D_6	0.510 ± 0.005	102.0
	Mean recovery (%)	99.0
	S.D. (%)	2.14
Test mixture (0.25 mg BTM + 0.25 mg DXM)[b]		
BTM	0.237 ± 0.001	94.5
	S.D. (%)	1.30
DXM	0.257 ± 0.001	102.8
	S.D. (%)	1.13

[a] $n = 3$.
[b] $n = 6$.

Further modification of the method by labelling the epimers with other chromophores or fluorophores is being investigated for the highly sensitive determination of the epimers in various samples of pharmaceutical, clinical or forensic interest.

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