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Short communication

HPLC analysis of 5*H*-benzo[a]carbazole with antifungal activity

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Abstract

A sensitive and simple high-performance liquid chromatographic (HPLC) method for the assay of 6,11-dihydro-2-methoxy-5*H*-benzo[a]carbazole (**1**) and 6,11-dihydro-2-methoxy-11-[2-(1-piperidinyl)]ethyl-5*H*-benzo[a]carbazole (**2**) was developed. The procedure is based on the use of the reversed-phase high-performance liquid chromatographic (RP-HPLC) method with UV detector. Each analysis required no longer than 11 min. A linear relationship between the concentration of both the drugs and the UV absorbance at 254 nm was obtained. This linearity was maintained over the concentration ranged from 5 to 80 μg/ml. The detection limits were found to be 1.6 and 0.7 ng for compounds **1** and **2**. The quantitation limits were found to be 5.3 and 2.5 ng for compounds **1** and **2**, respectively. For recovery studies, several determinations were carried out. Recovery values ranged from 98 to 102.1% for compound **1** and from 98.4 to 101.6% for compound **2**. Method precision was also evaluated and RSD% found was less than 2%. This method was applied without any interference from degradation products.

Keywords: Benzo[a]carbazole; RP-HPLC; Validation

1. Introduction

Benzodihydro[a]carbazoles have been reported as starting compounds for the synthesis of various drugs and possess important biological, pharmacological and medicinal activities [1–10]. Previously we have described some compounds with this structure, which demonstrated antimicrobial and antifungal activity [7,8]. From 16 new *N*-alkylated dihydro[a]benzocarbazoles tested in 1996 [7], exhibiting activity on gram-positive bacteria but not on gram-negative bacteria: 6,11-dihydro-2-methoxy-5*H*-benzo[a]carbazole (**1**) [181704-70-1] and 6,11-dihydro-2-methoxy-11-[2-(1-piperidinyl)]ethyl-5*H*-benzo[a]carbazole (**2**) [181704-71-2] (Fig. 1) were able to completely inhibit the growth of *C. albicans* below concentration of 4 μg/ml. Both compounds can be considered active on fluconazole-susceptible and fluconazole-resistant candida [8].

This method was developed for determining the purity of the synthesized compounds. This is the

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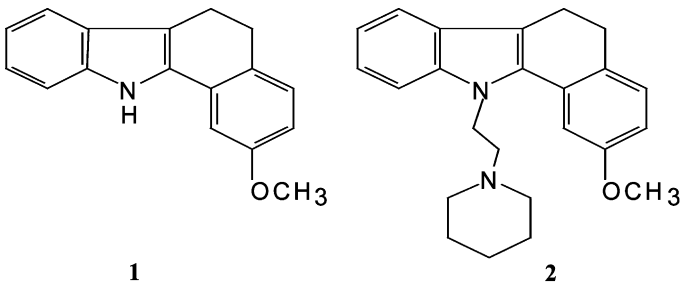

1 2

Fig. 1. Structures of antimicotics used in this study.

first method described for this type of compounds. As 6,11-dihydro-2-methoxy-5*H*-benzo[a]carbazole (**1**) could be an impurity of 6,11-dihydro-2-methoxy-11-[2-(1-piperidinyl)]ethyl-5*H*-benzo[a]carbazole (**2**), we developed this method for both drugs together.

As drug **2** has a basic functional group, the addition of a tertiary amine such as triethylamine is enough to improve peak shape.

2. Experimental

2.1. Chromatographic conditions

Analytical studies were carried out on a dual piston reciprocating Spectra Physics pump (Model ISO Chrom. LC pump), an UV–Vis Hewlett–Packard detector (Model 1050), a Hewlett–Packard integrator (Series 3395) and a Rheodyne injector (Model 7125). The analytical column was a Varian MICROSORB-MV™ 100 A C18 (4.6 × 250 mm, 5 μm) column. The solvent system consisted of methanol:water:triethylamine (80:20:0.3, v/v/v) and the pH was adjusted to 4.5 ± 0.1 with 85% phosphoric acid; allowed to equilibrate to room temperature and vacuum degassed before use. All analyses were performed under isocratic conditions at a 1.5 ml/min flow rate, and 15 min run time, at room temperature. The volume of each injection was 20 μl. Peaks were detected at 254 nm. Detector sensitivity was set at 1 a.u.f.s. In these conditions, 6,11-dihydro-2-methoxy-5*H*-benzo[a]carbazole retention time (t_R) was roughly 8 min and for 6,11-dihydro-2-methoxy-11-[2-(1-piperidinyl)]ethyl-5*H*-benzo[a]carbazole was 4 min.

2.2. Chemicals and reagents

Methanol used in the mobile phase was high-performance liquid chromatographic (HPLC) grade. Distilled water was passed through a 0.45 μm membrane filter. Triethylamine was AR grade. Solutions and mobile phase were prepared just before the use, and all solvents and solutions for HPLC analyses were filtered through a Micron Separations N04SP04700 nylon membrane filter (pore size of 0.45 μm) and vacuum degassed before use. Drugs were obtained by synthesis as described [7] and purified by recrystallization.

2.3. Procedure

Solutions were prepared on a weight basis and volumetric flasks were used as suitable containers in order to minimize solvent evaporation and to have a reference for its amount.

Before use and in order to evaluate the chromatographic system, a number of system suitability tests were performed. Prior to running system suitability checks, the column should be equilibrated for at least 30 min with the mobile phase flowing through the system. Peak identification was based on retention times while peak quantification was based on the external standard method. Each solution was injected in triplicate and the relative standard deviation (RSD) was required to remain below 2.5% on both drugs peak area basis. Both standards were interspersed with the samples if large number of analyses were to be performed.

2.3.1. Standard solutions and calibration curves

For both drugs, a standard stock solution of 0.1 mg/ml was prepared in methanol. The standard preparation was obtained by diluting the standard stock solution with mobile phase to yield a concentration of 0.05 mg/ml.

The standard curve for the assay covered the range of concentrations from 5 to 80 μg/ml and was prepared in the mobile phase from the standard stock solution. The calibration curve was constructed by plotting peak areas against micrograms injected.

2.3.2. System suitability

The analytical column was equilibrated with the eluting solvent system used. After an acceptable stable baseline was achieved, the standards and then the samples were analyzed. System suitability results were calculated according to the USP 24 ⟨6 2 1⟩ from typical chromatograms [11]. Instrument precision as determined by six successive injections of the standard preparation should provide an RSD below 1.0% for both drugs. Peak asymmetry or tailing factor, T, was calculated as $T = W_{0.05}/2f$; where $W_{0.05}$ is the distance from the leading edge to the tailing edge of the peak, measured at 5% of the peak height from the baseline and f is the distance from the peak maximum to the leading edge of the peak. Peak asymmetries were found to be 1.0 and 1.2 for **1** and **2**, respectively. Column efficiency should be greater than 300 theoretical plates. The resolution between **1** and **2** should be greater than 2.0.

2.3.3. Stability-indicating validation

The HPLC method was validated as stability indicating by forced degradation of the drugs. Samples were prepared by transferring approximately 5 mg of sample into 50 ml volumetric flask. Drugs were subjected to thermal (in an oven at 110 °C, 24 h) and photochemical degradation (in an open container exposed to daylight for 24 h). Intentional degradation was attempted using 10 ml of HCl 1 N, NaOH 1 N, H_2O_2 30 vol., H_2O and refluxing for at least 30 min. After the completion of degradation treatments, samples were allowed to cool to room temperature and diluted to the same concentration as the standard preparation, after being neutralized with acid–base if required. Samples were then analyzed against the standard.

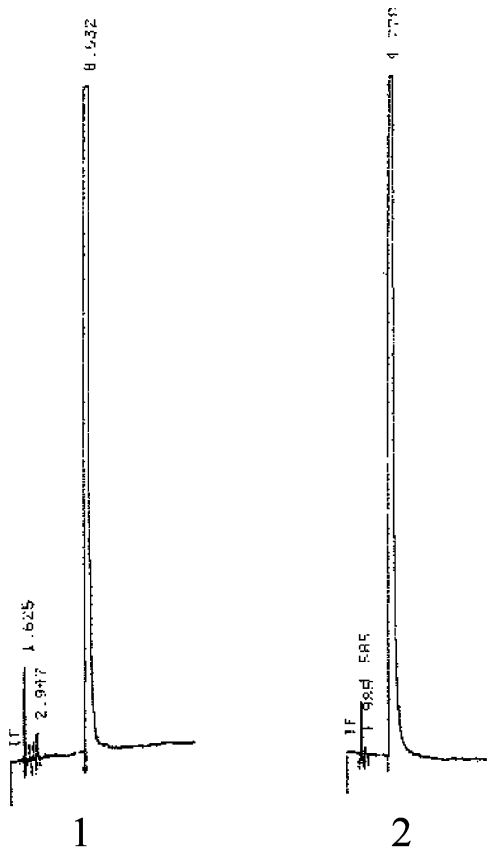

Fig. 2. Chromatograms: 1 (compound **1**) and 2 (compound **2**).

2.3.4. Precision

Instrument precision was evaluated by performing six consecutive injections of standard solution for both drugs. Method precision was evaluated by six repeated assays of the drug substance for both drugs.

2.3.5. Accuracy

Assay accuracy was assessed by preparing five solutions at 80, 90, 100, 110 and 120% of a standard and the amount recovered was determined.

Table 1

Condition	Time (h)	Recovery (%)	RRT of degradation products
*Degradation of 6,11-dihydro-2-methoxy-5H-benzo[a]carbazole (**1**)*			
Acid 1 N HCl, refluxed	0.5	73.9	0.28, 0.35, 1.90
Base 1 N NaOH, refluxed	0.5	88.0	0.21, 0.26, 0.37, 0.49, 1.55
H_2O_2 100 vol., refluxed	0.5	84.2	0.23, 0.28, 0.30, 0.35, 0.46
H_2O, refluxed	0.5	94.6	0.35
Heat dry, 110 °C	24	43.4	0.34, 1.19
Daylight	24	99.7	0.34
*Degradation of 6,11-dihydro-2-methoxy-11-[2-(1-piperidinyl)]ethyl-5H-benzo[a] carbazole (**2**)*			
Compound **1**			1.78
Acid 1 N HCl, refluxed	0.5	99.2	0.38, 0.42, 0.49
Base 1 N NaOH, refluxed	0.5	18.5	0.34, 0.36, 0.37
H_2O_2 100 vol., refluxed	0.5	30.0	0.34, 0.37
H_2O, refluxed	0.5	26.2	0.35
Heat dry, 110 °C	24	100.0	None detected
Daylight	24	99.2	None detected

RRT, relative retention time of each compound.

Table 2
Linearity data

Compound (%, w/w)	6,11-Dihydro-2-methoxy-5*H*-benzo-[a]carbazole (**1**)			6,11-Dihydro-2-methoxy-11-[2-(1-piperidinyl)]ethyl-5*H*-benzo[a]carbazole (**2**)		
	Injected (μg)	Average peak area response	RSD (%)	Injected (μg)	Average peak area response	RSD (%)
10	0.10	3272945	2.4	0.10	1591626	1.7
30	0.30	88582277	1.7	0.31	4429764	0.1
60	0.60	18012395	1.7	0.61	8920642	1.2
80	0.80	23980117	0.2	0.82	11717099	0.3
100	1.00	30039131	0.1	1.02	15140477	0.7
120	1.20	35595595	0.1	1.22	17696619	0.6
160	1.60	47925920	0.1	1.63	23970517	0.5
	Slope[a]	Intercept[b]				
Compound **1**	29932410 ± 68612498	120778 ± 64049999				
Compound **2**	14656565 ± 33660761	−31749 ± 32025268				

Compound **1**: $Y = 2.99 \times 10^7 + 1.2 \times 10^5$.Compound **2**: $Y = 1.47 \times 10^7 - 3.2 \times 10^4$.
[a] Confidence limits of the slope ($P = 0.05$).
[b] Confidence limits of the intercept ($P = 0.05$).

2.3.6. Lowest limit of detection (LOD) and quantitation (LOQ)

Serial dilutions of compounds **1** and **2** sample solution in mobile phase were performed in concentrations ranging from 0.4 to 4 μg/ml for compound **1** and 0.5 to 1.6 μg/ml for compound **2**.

3. Results and discussion

Typical chromatograms from compounds **1** and **2** are shown in Fig. 2. Forcing degradation of **1** and **2** was used to demonstrate the stability-indicating properties of the method. Degradation

Table 3
Accuracy results

Compound(%, w/w)	Amount added (mg)	Amount determined (mg)	Amount recovered (%)
*Compound **1***			
80	10.3	10.2	99.0
90	11.7	12.1	103.6
100	12.8	12.8	100.0
110	14.1	14.3	101.5
120	15.0	15.2	101.1
*Compound **2***			
80	10.1	10.4	103.0
90	11.3	11.5	102.0
100	12.7	12.7	100.0
110	13.8	13.7	99.6
120	15.2	15.7	103.0

Table 4
Analytical data for the determination of detection and quantitation limits

6,11-Dihydro-2-methoxy-5*H*-benzo-[a]carbazole (**1**)			6,11-Dihydro-2-methoxy-11-[2-(1-piperidinyl)]ethyl-5*H*-benzo[a]carbazole (**2**)		
Injected (μg)	Average peak area response	RSD (%)	Injected (μg)	Average peak area response	RSD (%)
0.00944	206464	12.0	0.0106	151233	1.0
0.0472	1087176	4.4	0.0212	308915	3.8
0.0944	2245329	3.9	0.0318	459957	0.7
Slope: 24020041			14562469		
Intercept: −29673			−2023		
Correlation coefficient: 0.9980			0.9989		

Compound **1**: $Y = 2.4 \times 10^7 - 3.0 \times 10^4$. Compound **2**: $Y = 1.46 \times 10^7 - 2.0 \times 10^3$.

was indicated in the stressed sample by a decrease of the expected value of the drug and increased levels of degradation products. No interfering peaks at the retention time of both the drugs were observed in any of the stressed sample. The presence of an alkyl substitution in compound **2** seemed to stabilize this structure when it is exposed to heat-dry. Although, compound **2** is less stable when it is exposed to base, water or oxidation degradation. The results of the stress study are presented in Table 1.

For six replicate sample preparations, 6,11-dihydro-2-methoxy-5*H*-benzo[a]carbazole results ranged from 98.0 to 102.1% with an RSD of 1.5%; 6,11-dihydro-2-methoxy-11-[2-(1-piperidinyl)]ethyl-5*H*-benzo[a]-carbazole results ranged from 98.4 to 101.6%, with an RSD of 1.1%.

Under experimental conditions described above, linear relationships were observed between the peak area of each compound versus the corresponding concentration, as shown in Table 2. The regression curve was calculated by the least-squares method. The correlation coefficients were 0.9999 for **1** and 0.9997 for **2**.

Accuracy defined as $M \pm (\text{S.D.}/\sqrt{n})t$ is $101.0 \pm 2.2\%$ for compound **1** and $101.5 \pm 2.0\%$ for compound **2**, where M is the mean potency value from recovery testing, S.D. is the standard deviation and the Student's t-test is $t(0.05, 4) = 2.776$ and n replicates (Table 3).

Method accuracy was demonstrated by plotting the amount determined (expressed in milligrams) against the amount added for both drugs. Linear regression analysis rendered slopes not significantly different from 1 (t-test, $P = 0.05$), intercepts were not significantly different from zero (t-test, $P = 0.05$), $r = 0.9956$ for compound **1** and $r = 0.9928$ for compound **2**.

The limits of detection (LODs) attained as $\mathrm{LOD}_{(k=3)} = k \times S_a/b$ (where b is the slope of the calibration graph and S_a is the S.D. of the blank signal) were found to be 1.6 and 0.7 ng for compounds **1** and **2**. The limits of quantitation (LOQs) were also attained as $\mathrm{LOQ}_{(k=10)} = k \times S_a/b$, and were found to be 5.3 and 2.5 ng for compounds **1** and **2**, respectively (Table 4).

4. Conclusion

The HPLC method described in this work is selective, reliable, sensitive and useful for stability studies on compounds **1** and **2**. Moreover, the proposed method offers a short analytical run time of 11 min and achieved a good resolution between compounds **1** and **2**.

Acknowledgements

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