DETERMINATION OF MOXONIDINE AND ITS IMPURITIES BY THIN-LAYER CHROMATOGRAPHY

Slavica Filipic[1], Musbah Salem Mohamed Shenger[1], Katarina Nikolic[1], Danica Agbaba[1]

[1]Department of Pharmaceutical Chemistry, University of Belgrade - Faculty of Pharmacy, Belgrade, Serbia

Address correspondence to Danica Agbaba, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11000 Belgrade, Serbia. E-mail: danica@pharmacy.bg.ac.rs

Abstract

A thin-layer chromatographic method for simultaneous determination of moxonidine and its four impurities was developed and validated. Separation of the examined compounds was performed on chromatographic plates precoated with silica gel 60 F_{254} and using methanol-toluene-dichloroethane-ammonia 2:3:3:0.1 (v/v/v/v) as mobile phase. Ascending development mode was performed in the twin-trough chromatographic chamber, which was presaturated with mobile phase vapors for 15 min. The developed chromatographic plates were dried in air and densitometrically scanned at the wavelengths 260 and 280 nm. Regression coefficients ($r \geq 0.998$), recovery (90.10 % - 107.63 %), LOQ of impurities (20 ng band^{-1} equivalent to the 0.12 % impurity level) and robustness were validated and found satisfactory. The developed method is well suited for quantitative analysis and purity control of moxonidine in its dosage forms.

KEYWORDS: impurities, moxonidine, method validation, pharmaceuticals, quantitation, TLC

INTRODUCTION

Moxonidine as a centrally active I_1 receptor agonist with minor activity at α_2-adrenoceptors has been extensively used in treatment of hypertension. Moreover, moxonidine improves metabolic profile of patients with hypertension and the type 2 diabetes, or with an impaired glucose tolerance.[1,2]

Moxonidine belongs to the second generation of imidazoline compounds and some theoretical studies have been performed on physicochemical properties of a series of structurally similar drugs acting on the I_1 and α_2-adrenoceptors.[3,4] Lipophilicity and acidity of moxonidine, and those of structurally similar imidazolines and oxazolines have been evaluated with aid of different separation techniques such, as high performance liquid chromatography (HPLC)[5] and thin layer chromatography (TLC).[6,7] This evaluation was based on measuring of retention behavior of the compounds of interest in the employed separation systems. Several reports are available describing determination of moxonidine in human plasma by means of liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS)[8] and gas chromatography-mass spectrometry (GC-MS).[9]

According to European Pharmacopoeia (EP)[10] and British Pharmacopoeia (BP),[11] determination of moxonidine and its four related substances is based on HPLC. Recently, the HPLC method [12] was developed and validated for the determination of moxonidine in the presence of its impurities, and the UPLC method[13] was devised as a stability indicating method for the determination of moxonidine and its degradation products in

pharmaceuticals. Hydrophilic interaction chromatography (HILIC) devised for the separation of moxonidine in the presence of five related compounds is an alternative to the reversed-phase (RP) HPLC for determination of polar analytes in a pharmaceutical matrix.[14]

The EP monograph of moxonidine in the section referring to the related substances focuses on the two process-related impurities only, i.e., impurity A (6-chloromoxonidine) and impurity B (4-metoxymoxonidine), whereas further two impurities, C (4-hydroxymoxonidine) and D (6-desmethylmoxonidine), are classified as *other* impurities. Structures of moxonidine and its impurities are shown in **Fig. 1.** The impurity types and ratios differ depending on the reaction conditions. Position C(4)/C(6) of the pyrimidine moiety is reactive to nucleophilic substitution.[15] Even a low level of humidity or the presence of other nucleophiles which might originate from different sources (such, as the tablet matrix/excipients) can affect the stability of moxonidine and generate impurities. A stability indicating high performance thin-layer chromatographic method was reported and validated for analytical estimation of moxonidine in the presences of the degradants, excipients and impurities. Structure of the impurities was not elucidated.[16.]

Except for the aforementioned reports,[12,13] there is still, however, a limited information on an assessment of the moxonidine purity in pharmaceuticals. This prompted us to develop and validate an alternative TLC method for the simultaneous determination of moxonidine and its impurities using instrumental planar chromatography.

EXPERIMENTAL

Materials

Moxonidine, 4-chloro-*N*-(imidazolidin-2-ylidene)-6-methoxy-2-methylpyrimidin-5-amine; **Impurity A**, 4,6-dichloro-*N*-(imidazolidin-2-ylidene)-2-methylpyrimidin-5-amine (6-chloromoxonidine); **Impurity B**, *N*-(imidazolidin-2-ylidene)-4,6-dimethoxy-2-methylpyrimidin-5-amine (4-methoxymoxonidine); **Impurity C**, 5-[(imidazolidin-2-ylidene)amino]-6-methoxy-2-methylpyrimidin-4-ol (4-hydroxymoxonidine,); and **Impurity D,** 6-chloro-5-[(imidazolidin-2-ylidene)amino]-2-methylpyrimidin-4-ol (6 desmethylmoxonidine) were obtained from Chemagis (Bnei Brak, Israel). The Moxogamma® 0.4 mg filmtablets were manufactured by Worwag Pharma (Böblingen,Germany).

Lactosa monohydrate, povidone K-25, crospovidone, and magnesium stearate were of the EP quality and used for the preparation of the placebo mixture. All other reagents, i.e., toluene (POCH, Gliwice, Poland), 1,2 dichloroethane (Fisher Chemical**,** Loughborough**,** UK), ammonia solution 25% (Merck, Darmstadt,Germany) and methanol (Merck, Darmstadt, Germany) were of analytical purity.

Solutions

Standard Solutions

Stock solutions for moxonidine (1 mg mL^{-1}) and impurities A, B, C and D (0.2 mg mL^{-1}) were prepared in methanol. For the calibration curves, six different solutions were

prepared by diluting the stock solutions in the concentration range from 0.2 to 0.6 mg mL^{-1} for moxonidine and 0.02 to 0.2 mg mL^{-1} for impurities A, B, C and D.

Sample Solutions

Ten tablets from which the film was previously removed were weighed and pulverized. The quantity of the powdered tablets equivalent to 2.0 mg moxonidine was transferred to the 5 mL volumetric flask and sonicated in 4 mL methanol for 20 min, using an ultrasonic bath. The solution was made up to 5 mL with the same solvent, and then centrifuged at 3000 U/min for 15 min. The obtained supernatant was filtered through the 0.45 mm pore size membrane filter (Millipore). For an assay of moxonidine and the impurities, the 1-µL and 40-µL aliquots of the filtrates, respectively, were applied to the chromatographic plates.

Chromatography

TLC was performed on the 20 cm × 10 cm plates cut from the 20 cm × 20 cm aluminium backed plates, precoated with silica gel 60 F_{254} (Merck, Darmstadt, Germany).

Standard and sample solutions were applied 10 mm above the lower edge of the plate, using the Linomat 5 (Camag Muttenz, Switzerland) application device. Samples were applied band-wise with the 10 mm band width, with an application rate of 100 nL s^{-1}. Ascending development mode at ambient temperature was performed to the distance of 95 mm in the twin-trough TLC chamber presaturated with mobile phase (methanol-toluene-dichloroethane-ammonia, 2:3:3:0.1 (v/v/v/v)) for 15 min. After the development,

the chromatographic plates were dried for 30 minutes at ambient air and once again developed to the distance of 95 mm in the freshly prepared mobile phase of the same composition as before, after 15 min chamber presaturation. Dried chromatographic plates were scanned at the wavelengths of 280 and 260 nm by means of the Camag TLC Scanner II in the reflectance/absorbance mode.

RESULTS AND DISCUSSION

In order to optimize chromatographic conditions for an efficient separation of moxonidine and its four impurities, different stationary phases and different mobile phase compositions were examined. Preliminary studies have started from examination of the retention behavior of the analytes using single nonpolar (toluene) and single polar (methanol) solvent as two monocomponent mobile phases, and the polar silica gel plates as stationary phase.

In contrast to the retention behavior in nonpolar solvents (such, as toluen), where all analytes demonstrated high affinity toward stationary phase and were retained on the start line, higher mobility (especially with moxonidine) was observed with use of a polar solvent (such, as methanol). In order to achieve satisfactory resolution of the examined compounds, further tests were directed toward examination of the analytes' retention in the toluene-methanol mixture. In order to reduce peak tailing, basic solvent (ammonia, or triethylamine (TEA)) was added to mobile phase . Higher volume fraction of methanol in mobile phase resulted in a too high R_f value for moxonidine and impurity A, while impurities B, C and D were retained close to the start line. Therefore, higher proportion

of toluen was selected, which significantly reduced the R_f values for moxonidine and increased resolution among the tested compounds. Different volume ratios of methanol, toluen and TEA were tested to optimize the mobile phase composition and in the course of these experiments, considerable difference in the retention behavior was observed between impurity C and impurity A. In fact, impurity C remained close to the start line, while impurity A migrated close to the front line.

Well separated and compact zones were obtained by adding dichloroethane to the eluent mixture and the next qualitative and quantitative mobile phase composition was assumed as methanol-toluene-dichloroethane-TEA, 2:3:3:0.1, v/v/v/v. In that case, the migration distances (MD ± RSD) for moxonidine and impurities A, B, C and D were equal, respectively, to 41.2 mm ± 0.99 %, 51.9 mm ± 0.82 %, 24.2 mm ± 0.40 %, 13.6 mm ± 0.87 %, and 19.4 mm ± 0.74 %. Later it was noticed that impurities C and A co-eluted with the tablet matrix, which was finally avoided by replacing TEA with an equal volume proportion of ammonia and assuming double development of the chromatogram using the same mobile phase, methanol-toluen-dichloroethane-ammonia 2:3:3:0.1, v/v/v/v. Thereby the method specificity was achieved and the migration distances for moxonidine and impurities A, B, C and D were: 58.5 mm ± 0.78 %, 64.4 mm ± 0.95 %, 34.5 mm ± 0.56 %, 16.1 mm ± 0.68 %, and 25.6 mm ± 0.85 %.

Retention order of the separated substances (C>D>B>moxonidine>A) is basically driven by structural characteristics of the C4/6 pirimidine moiety present in the investigated compounds and by an ability of these compounds to form hydrogen bonds with the

siloxane and silanol groups of silica gel. Similar elution order of moxonidine and its impurities was observed in the polar HILIC HPLC system,[14] with one exception only for the reverse order of D and C (D>C>B>moxonidine>A).

Testing the elaborated mobile phase composition upon the HPTLC and HPTLC LiChrospher Si60 plates, no better resolutions was achieved, so that the aluminium backed chromatographic plates precoated with silica gel 60 F_{254} were used for further validation of the developed TLC method.

For moxonidine and impurities A, C and D, quantitative measurements were performed at the wavelength 280 nm, and for impurity B, at the the wavelength 260 nm. The relationship between the peak area and the amount of the applied substance was evaluated with use of the linear and the second degree polynomial regression functions. Fitting with the second-degree polynomial was done because a wider concentration range is required for quantification of an impurity in the purity method. The obtained regression data are summarized in **Table 1**.

To avoid systematic errors, an effect of larger amounts of moxonidine on the peak shape and resolution of impurities had to be tested. Method accuracy was therefore verified by determination of impurities A, B, C and D in the presence of moxonidine. The laboratory-made placebos were spiked with moxonidine and a mixture of 0.3, 0.5, and 1.2 % impurities A, B, C and D, respectively. The application volumes were 40-µL and 20-

µL for the estimation of 0.3, 0.5 and 1.2 % impurities, respectively. Scanned profiles obtained for the moxonidine samples spiked with impurities are presented in **Fig. 2.**

Calculated recoveries were plotted against the expected values (corresponding to the standards without moxonidine). Recoveries and the relative standard deviation (RSD) values for all impurities was acceptable for the purity method (Table 2). Repeatability of the method was evaluated by chromatographic replicate applications (n=6) of moxonidine and impurities A, B, C and D at three different concentrations. Statistical data obtained from these experiments are given in Table 3.

The limit of detection (LOD) and the limit of quantification (LOQ) values were obtained experimentally and statistically. Experimentally obtained LOD values for impurities A, B, C and D were equal to 7 ng per band, while experimentally obtained LOQ values were equal to 20 ng per band (corresponding with the impurity levels of 0.04 % and 0.12 %, respectively). Statistically, the LOD values were determined by fitting the interday back-calculated standard deviation for each calibration standard. The y-intercept was then equal to SD_0 (the estimated standard deviation at zero concentration). LOD was defined as $3SD_0$ and LOQ as $10SD_0$. The LOD values obtained for impurities A, B, C and D were 8.41 ng, 7.89 ng, 7.32 ng, and 3.73 ng, respectively (equivalent to the impurity levels of 0.053 %, 0.049 %, 0.046 %, and 0.023 %, respectively). The LOQ values for impurities A, B, C and D were 28.00 ng, 26.30 ng, 24.41 ng, and 12,43 ng, respectively (equivalent to the impurity levels of 0.175 %, 0.164 %, 0.153 %, and 0.078 %, respectively).

Robustness is a measure of the capacity of the method to remain unaffected by small yet deliberate variations of working conditions, and it is indicative of the method reliability.[17] In the robustness test, the effects of different amounts of methanol in mobile phase (± 5%), different developing distances (90 and 98 mm), different spot band sizes (6 and 8 mm), and different chamber geometry (twin-trough and flat) were examined. Selection of the tested factors was based on our experience and observations made in the course of method development. Based on the obtained results, no observable effects on resolution between moxonidine and its impurities was perceived, showing that the proposed method remains unafected by small yet deliberate variations of the working conditions.

The method was used to screen the commercial dosage forms. The densitogram obtained for moxonidine and the impurity standards is shown in **Fig. 3**. No intereference of tablet formulation was observed using the developed chromatographic system, which confirms good selectivity of the method. The results obtained for the content of moxonidine of 97.41 % and the found levels of impurities C and D of 0.21 % and 0.26 %, respectively, meet the requirements of the manufacturer, and do not exceeded 1.0 %. The contents of impurities D and A were established as lower than LOD of the proposed method. (**Table 4**).

CONCLUSION

In spite of the fact that the number of TLC application is steadily decreasing, replaced by the HPLC methods,[18] the obtained results show that instrumental planar chromatography

is powerful enough to be used for the purity testing of the low dosage units (as it is the case with 0.4 mg moxonidine per tablet) and it is well suited as an alternative method for the drug quality control.

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TABLE 1. Statistical Data for the Calibration Curves-Calibration Function $y = a + bx + cx^2$

Compound	**Concentration range [ng band^{-1}]**	**a**	**b**	**c**	**SD**	**r**
Moxonidine	200-600	-22.83 ± 10.67	0.90 ± 0.06	1.24E-04 ± 6.42 E-05	4.306	0.999
Impurity A	20-200	77.66 ± 12.49	3.45 ± 0.29	-9.53E-04 ± 0.001	9.680	0.998
Impurity B	20-200	13.41 ± 32.15	8.06 ± 0.89	-0.007 ± 0.005	21.199	0.998
Impurity C	20-200	10.44 ± 16.86	5.35 ± 0.40	-0.003 ± 0.002	13.062	0.999
Impurity D	20-200	18.77 ± 7.62	4.75 ± 0.18	-0.004 ± 8.09E-04	5.906	0.999

TABLE 2. Accuracy of the Method

Compound	**Level [%]**	**Mean Recovery [%]**	**RSD [%]**
Moxonidine	80	99.52	1.38
	100	100.36	0.89
	120	99.81	0.95
Impurity A	0.3	103.28	5.89
	0.5	107.63	2.20
	1.2	101.07	2.37
Impurity B	0.3	98.29	2.48
	0.5	104.40	4.25
	1.2	97.87	2.06
Impurity C	0.3	100.37	2.96
	0.5	93.95	3.84
	1.2	90.10	3.87
Impurity D	0.3	95.84	1.78
	0.5	101.76	3.76
	1.2	95.66	2.07

TABLE 3. Precision of the Method (n=6)

Amount [ng band^{-1}]	**Moxonidine RSD [%]**	**Impurity A RSD [%]**	**Impurity B RSD [%]**	**Impurity C RSD [%]**	**Impurity D RSD [%]**
200	1.00				
400	0.74				
600	0.68				
50		2.32	3.89	3.74	2.71
80		1.40	2.63	1.44	1.75
190		1.81	1.50	1.29	1.19

TABLE 4. Assay of Moxonidine and Its Impurities

Sample	**Moxonidine [% ± RSD]**	**Impurity A [% ± RSD]**	**Impurity B [% ± RSD]**	**Impurity C [% ± RSD]**	**Impurity D [% ± RSD]**
Moxogamma® 0.4 mg	97.41 ± 1.92	n.d.	n.d.	0.21 ± 6.87	0.26 ± 4.82

Figure 1. Chemical structures of moxonidine and its impurities

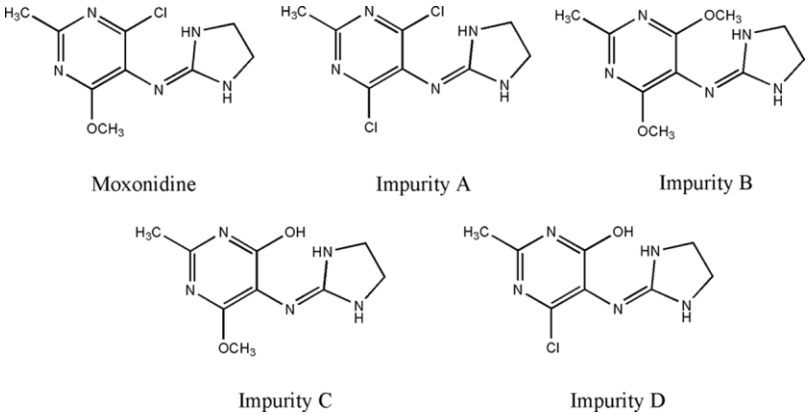

Figure 2. Densitograms obtained at the wavelength 280 nm for (a) sample of placebo; (b,d,f) standards of impurities A, B, C and D at 0.3 %, 0.5 % and 1.2 % level (peaks 4, 3, 1 and 2, respectively); (c,e,g) placebo spiked with moxonidine and impurities A, B, C and D at 0.3 %, 0.5 % and 1.2 % level (peaks 5, 4, 3, 1 and 2, respectively).

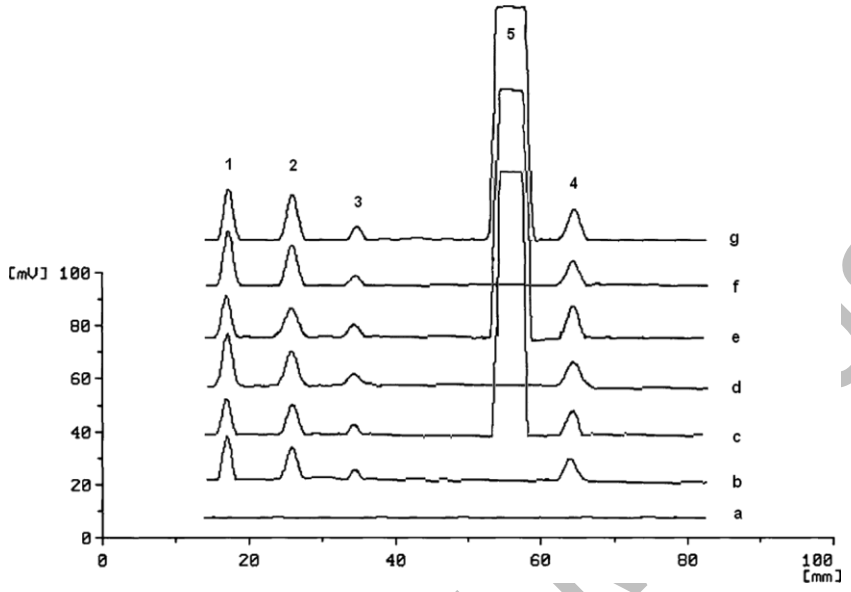

Figure 3. Densitogram obtained at the wavelength 280 nm for (a) sample of placebo; (b,c) samples of the moxonidine tablet; (d) standards of impurities A, B, C and D at 0.3 % level (peaks 4, 3, 1 and 2, respectively); (e) standards of impurities A, B, C and D at 0.5 % level (peaks 4, 3, 1 and 2, respectively).