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RESEARCH ARTICLE

DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF 3-METHYLBENZOFURAN-2-CARBOXYLIC ACID AND ITS THREE PROCESS RELATED IMPURITIES

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ABSTRACT

A simple, selective, accurate, precise and linear RP-HPLC method was developed and validation for simultaneous determination of 3-methylbenzofuran-2-carboxylic acid and its three process related impurities. The IUPAC name of impurities were impurity-A (1-(2-hydroxyphenyl) ethanone), impurity-B (3-methylbenzofuran-2-carbonitrile), impurity-C (3-methylbenzofuran-2-carboxamide). The good effective separation was achieved on a Reversed Phase High Performance liquid chromatography method with UV detection, Agilent SB C18 (250× 4.6 mm, 3.5µm) as stationary phase with binary gradient mode solvent phase A of buffer (pH adjust =3.4, with Acetic acid) and phase B of use acetonitrile. The Flow rate of the mobile phase was 0.9 ml/min. The developed method was validated in terms of system suitability and linearity level 150% (0.75µg/ml) to 0.5% (0.0025µg/ml) limits of detection and quantification the for 3MBCA and its four process related impurities short term and long term stability of the analysts in the prepared solution and robustness, following the ICH guidelines.

INTRODUCTION

3MBCA product categories are API (Anti-fungal, Antitumor, Anticancer) intermediates [1-4]. 3MBCA is a benzofuran based acid used in the preparation of potent, selective, and orally bio available Matrix Metalloproteinase (MMP) inhibitors for the treatment of osteoarthritis [1] and anti-tumor agents 4-hydroxy-3-methyl-6-phenylbenzofuran-2-carboxylic acid ethyl ester derivatives (Anti cancer drug) [2] and synthesis of antifungal agents targeting fungal drugs N-myristoyltransferase and antifungal activity of derivatives of 2 and 3 benzofuran carboxylic acid [3-4]. Understanding the fate and origin of organic impurities within the synthesis process along with a very good control strategy is an integral part of the quality control of intermediate and drug products. This approach requires an aggressive analytical and chemical search for potential impurities in the key raw materials, intermediates and drug substance, and experimental studies to track their fate through the synthesis process in order to understand the process capability for rejecting such impurities.

The application of various analytical techniques (HPLC, LC-MS, NMR, etc) and development of sensitive and selective methods for impurity detection, identification separation and quantification [5-7]. Many route to synthesis process of 3MBCA from 1-(2-hydroxyphenyl) ethanone and 3-methylbenzofuran-2-carbonitrile and 3-methylbenzofuran-2-carboxamide, [8-17] Structures show in Fig.1. 3MBCA Chemical formula is $C_{10}H_8O_3$, molecular mass 176.2 gm/mole and IUPAC Name 3-methylbenzofuran-2-carboxylic acid. 3MBCA and its three process related impurities. The IUPAC name of impurities were impurity-A (1-(2-hydroxyphenyl) ethanone), Chemical formula $C_8H_8O_2$, molecular mass 136.1 gm/mole, impurity-B (3-methylbenzofuran-2-carbonitrile), Chemical formula $C_{10}H_7NO$, molecular mass 157.2 gm/mole, impurity-C (3-methylbenzofuran-2-carboxamide), Chemical formula $C_{10}H_9NO_2$, molecular mass 175.2 gm/mole, Structures show in Fig.1.

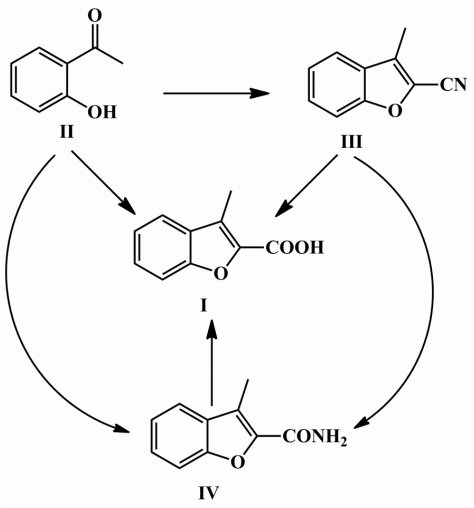

Figure 1 Reference [8-17] synthetic route of 3MBCA from raw materials, intermediate stage and impurities.

I: Structure of the 3MBCA, II: Structure of the Impurity A, III: Structure of the Impurity B, IV: Structure of the Impurity C.

MATERIALS AND METHODS

Instrumentation and Software

The HPLC system of Agilent HPLC 1100 series variable wavelength detector (VWD), The Diode Array Detector (DAD) Microprocessor, quaternary pump, Agilent Technologies international sarl 1100 series, auto sample, micro auto sample, preparative auto sample, Thermostatic column compartment, used for this entire study and chromatographic separation was achieved on Agilent SB C18 (250× 4.6 mm, 3.5µm) as stationary phase with binary gradient mode. Balance: Analytical balance, pH Meter, 0.45µ membrane filters.

Chemicals and Reagents

The pharmaceutical grade intermediate of 3MBCA (99.9% pure) and its three process related impurities (99.9% pure) were procured from market. All solvents used in the present study i.e. acetonitrile, Milli-Q water, ammonium acetate, acetic acid and other chemicals used for the analysis were of analytical grade procured from Merck & SD Fine Ltd. India.

Details of Method Chromatographic Conditions

Agilent SB C18 column (250× 4.6 mm, 3.5µm) was used as stationary phase with binary gradient mode solvent. Diluents preparation: acetronitrile: Milli-Q water in ratio of 60:40 v/v. Tablets are included in Table 1.

Table 1 Details of Method: Chromatographic condition

Column	Agilent SB C18 (250× 4.6 mm,3.5µm)		
Buffer	composed of 0.8 gm Ammonium acetate in 1000ml water buffer (P^H adjust =3.4, with Acetic acid) mix it well and filter through 0.45µ membrane filter		
Mobile phase-A	Buffer		
Mobile phase-B	filter and degas Acetronitrile		
Diluent	Acetronitrile: Milli-Q water in ratio 60:40 v/v		
Column oven temp. °C	35°C		
Detection	UV at 240 nm		
Injection volume	10.0 µl		
Run time	40.0 minutes		
Flow rate	0.9 ml/ min		
Gradient Programme	Time(min)	Mobile Phase-A %	Mobile Phase-B %
	0.1	60	40
	40	25	75

Preparation of Solutions

Specificity solution for Retention time confirmation and system suitability solution

Accurately weighted 10 mg of each 3MBCA, impurity A, impurity B and impurity C into 100 ml volumetric flask, dissolve in 30 ml diluent and sonicate to dissolve in ultrasonic water bath and dilute up to the mark with diluent.

Test solution

Accurately weighted 15.0 mg of test sample (3MBCA) into 50 ml volumetric flask dissolve in 5 ml diluent and sonicate to dissolve in ultrasonic water bath and dilute up to the mark with diluent.

Solution preparation

ID Solution of impurity A

Weighed accurately and transferred about 25mg of impurity A (standard) into a 100 ml volumetric flask, added 10 ml diluent and sonicate to dissolve in ultrasonic water bath and diluted up to the mark with diluent. The sample concentration was 250ppm.

ID Solution of impurity B

Weighed accurately and transferred about 25mg of impurity B (standard) into a 100 ml volumetric flask, added 10 ml diluent and sonicate to dissolve in ultrasonic water bath and diluted up to the mark with diluent. The sample concentration was 250ppm.

ID Solution of impurity C

Weighed accurately and transferred about 25mg of impurity C (standard) into a 100 ml volumetric flask, added 10 ml diluent and sonicate to dissolve in ultrasonic water bath and diluted up to the mark with diluent. The sample concentration was 250ppm.

Impurity composite stock solution

Transferred 3.0 ml of each ID solution of impurity A, impurity B, impurity C, to 100ml volumetric flask make volume up to mark with diluent. Sample concentration was 7.5ppm each impurity.

Test solution spiked with impurities at specification level

Weighed accurately and transferred about 25 mg of test sample (3MBCA) into a 50 ml volumetric flask, added 10 ml diluent and sonicated it to dissolve the content. Added 5.0 ml of impurity composite stock solution and diluted up to the mark with diluent and mix. Test sample (3MBCA) concentration is 500ppm and sample concentration is 0.75ppm each Impurity.

METHOD VALIDATION

Validation of the developed method for the determination of 3MBCA and its three process related impurities. was performed according to the ICH guidelines [18-19] with standards bulk drug thus system suitability along with method selectivity, specificity , linearity, range, precision (repeatability) and intermediate precision, accuracy, limits of detection and quantification. Method validation includes several parameters have been performed systematically on HPLC instruments as per ICH guidelines.

Limit of Detection and Limit of Quantification

Study design: Determine the LOD and LOQ for impurity A, impurity B, impurity C and 3MBCA. Injected solution as per the following sequence. Evaluated the data and drawn a linearity plot from the level, which detected to 150.0 % of specification limit. Procedure of inject blank (diluent), system suitability solution for retention time conformation, linearity level 150% (0.75 µg/ml) to 0.5% (0.0025 µg/ml) of specification limit.

The limit of quantification (LOQ) and limit of detection (LOD) were estimated using the following equations

$$LOQ=10\,б/s \text{ and } LOD=3.3б/s$$

Where б is the standard deviation of intercept and s is the slope of the calibration curve. Standard stock solution:

Weighed accurately and transferred about 25 mg of 3MBCA and each impurity A, impurity B, impurity C into a four different 100 ml volumetric flask. Added 10 ml diluent into each of the flask and sonicate to dissolve the content. Dilute

up to the mark with diluent and mix. The sample of 3MBCA concentration is 250ppm and sample concentration is 250ppm each impurity.

Standard stock solution for linearity

Transfer 3.0 ml of each impurity A, impurity B and impurity C standard stock solution and 2.0 ml of 3MBCA Standard stock solution into 100 ml volumetric flask. Dilute up to the mark with diluent and mix. The sample of 3MBCA concentration is 5ppm and sample concentration is 7.5ppm each impurity. Procedure of Inject sequence into the chromatographic system linearity level 150% (0.75µg/ ml) to 0.5% (0.0025µg/ml) of specification limit. Evaluate the chromatogram. Record the area of analyte. Calculate the establish the LOQ and LOD for the test method.

Precision, Accuracy, Robustness, Stability of analytical solution study

Method validation includes Precision, Accuracy, Robustness, Stability of analytical solution all study parameter have been performed on HPLC instrument as per ICH guideline Q2A and Q2B [18-19]. The Relative Standard Deviation (RSD) were estimated using the following equations.

$$RSD = (100\times SD)/AVG$$

Where AVG is the number of results in AVERAGE result, SD is the Standard Deviation.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The development of HPLC methods for determining drugs has received great attention in analytical research due to its use in quality control. The technique is unique, versatile, universal and basic. It is easy to operate and is also well-utilized by researchers. The main objective of method development is to determine the 3MBCA and three process related impurities (A, B, C) of synthesis of drugs. The presence of the similar property compound impurity (ABC) and 3MBCA among the other constituents that necessitated an almost aqueous mobile phase led to the selection of UV detection separation was achieved on Agilent SB C18 (250× 4.6 mm, 3.5µm) as stationary phase with binary gradient mode solvent or equivalent column for the development of the proposed method. impurity A, B and 3MBCA well separate but the closely nature of impurity C (amide group) and 3MBCA(carboxylic acid group) two compound highly closely polarity and the relatively similar behavior of 3MBCA demanded extensive optimization of the pH of the mobile phase A and the need of and a gradient elution. The selection of the pH of the mobile phase was very critical for pH=3.4, the peaks of the acidic substances 3MBCA and impurity C would move the right, closer to the 3MBCA peak for pH=3.4, these two substances would co elute and move to the left of the chromatogram. Finally a linear gradient program with an initial mobile phase A composed of 0.8 gm Ammonium acetate in 1000ml water buffer (pH was adjusted 3.4 by using Acetic acid) mixed well and filtered through 0.45µ membrane filter. Mobile B preparation: Use filter and degas of acetronitrile, and acceptable separation of the three impurities and 3MBCA. The wavelength variation of the detector during the analysis was based on the maximum wave length (max) absorbance of the relevant compounds. 3MBCA and impurity C absorbed below 254 nm and impurity A, B absorbed above 240 nm. The wavelength was set at the initial value of 240 nm in order prepare the system for the next injection. The optimal wavelength scheme along with details is given in section 2.3. A typical chromatogram of the separation of the two analytes under these conditions is presented in Fig. 2.

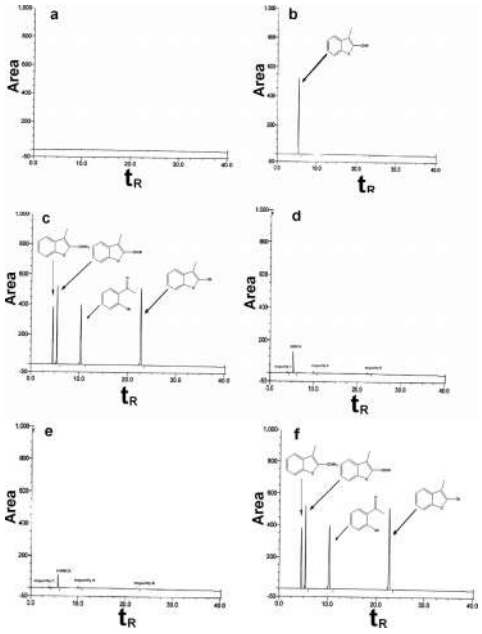

Figure 2 Chromatograph for method development and system suitability of 3MBCA and impurity A,B,C. Chromatograph of (a) diluent,(b) reference solution,(c) System suitability solution,(d) LOD solution,(e) LOQ solution (f) test solution spiked with impurity A,B,C

METHOD VALIDATION

System Suitability

The system suitability test is very important because it ensures the validity of the analytical procedure. In the present work a mixed standard, consisted of 3MBCA and the three impurity (described in section 2.5.1) was the system suitability solution used daily for this purpose. Thus the consistency of the retention time of 3MBCA (t_R) and the area of the relative retention times (Rt_R), and resolution (R_S) between adjacent peaks, were the critical parameters examined every day during the validation of the method the critical parameters of R_S met the acceptance criteria ($R_S \geq 1.5$,) and assured for a good separation quantification .

Linearity and range

Table 1 presents the analytical parameters of typical standard calibration curves of 3MBCA and its three impurity in diluents. All calibration curves for 3MBCA presented coefficient of determination R^2>0.9999, while for the impurity A, B, C of R^2 was greater than 0.995 as required. A lack-of-fit test was performed for all calibration curves and the calculated R^2-values of the representative curves included in table 2 were the following at 96% confidence level for 3MBCA correlation 0.999, the impurity A,B,C was correlation 0.999,0.999,0.999 system suitability parameter was comply. The correlation coefficient would not be less than 0.98. Y- Intercept ≤25% referred to the calculated response of the x-value corresponding to the concentration of the specification limit. Shown in and Fig. 3.Representative Linearity and range results in memory tablets are included in table 2.

Table 2 Linearity, t_R (retention time), Coefficient of determination (R^2), Y-intercept, LOD and LOQ data for proposed Method of 3MBCA and impurity A, B, C

Substance	Range (µg/ml)	t_R,(retention time)	Rt_R, (relative time)	Coefficient of determination (R^2)	Y-intercept	LOQ	LOD	Relative Response Factor
3MBCA	0.0025-0.75	5.42	1	0.999	152.×+14.36	0.0053	0.0017	
Impurity A	0.00375-1.12	10.33	1.91	0.999	303.×+115.7	0.0015	0.00050	1.25
Impurity B	0.00375-1.12	22.69	4.19	0.999	383.×+32.34	0.013	0.0045	1.55
Impurity C	0.00375-1.12	4.39	0.81	0.999	268.×+95.13	0.008	0.0027	1.09

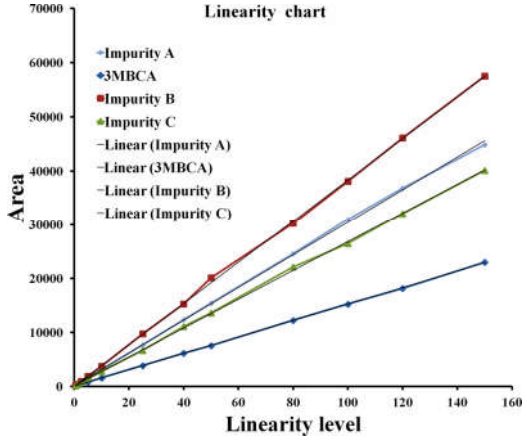

Figure 3 Plot of Area verses linearity level (0.5% to 150%) and confidence level for 3MBCA correlation (R^2) 0.999, Y- Intercept 152.× +14.36,and Impurity A,B,C .The correlation Coefficient would not be less than 0.995. Y- Intercept ≤25% referred to the calculated response of the x-value corresponding to the concentration (linearity level) of the specification limit.

Precision and accuracy

The repeatability and intermediate precision were expressed as the % relative standard deviation (% RSD) of each analyte concentration using one way 3MBCA. The results are presented in table 3.

Accuracy study

Accuracy of the test method was be demonstrated by spiking impurity A,B,Cand 3MBCA in a test sample at LOQ, 50%,100%,150% level of target concentration. Recovery to RSD values of 3MBCA and its Process three impurity have been found in the following ranges, 3MBCA 0.472-1.570, Impurity A (0.50-2.65), Impurity B (0.63-3.22), Impurity C (0.377-3.77) Representative recovery results in bulk drug intermediate are included in table 3;System suitability parameter was comply. and % RSD obtained from three sets was be NMT 10.0.Aceeptabble for recovery LOQ level (96.71%-97.91%) and 50%,100%,150% level (98.99 % to 100.1%).Acceptable criteria for recovery study results in bulk drug intermediate are included in table.3 .

Stability

At the beginning of the method development it was observed that 3MBCA stock solutions prepared in diluent (acetonitrile: water; 60:40) seemed to be very unstable and additional peaks appeared in the chromatograms. Therefore based on this observation a stability study on 3MBCA stock solution in diluent at RT and 5°C for 24 hrs, 48hrs followed which they were stable as shown in figure.

Table 3 Accuracy and Recovery and Precision data for proposed method of 3MBCA and Impurity A, B, C

Substance	Level of %	Recovery Data		Accuracy Data		Precision Data			
		Mean	RSD	Mean	RSD	SD		RSD	
						1 Day	2 Day	1 Day	2 Day
3MBCA	150	99.88	0.998	256.8	1.068	83656	82848	0.534	0.529
	100	99.56	0.996	70.06	0.472				
	50	99.01	0.990	87.12	1.110				
	LOQ	97.30	0.973	15	1.570				
Impurity A	150			224.42	0.50	575	828	1.90	2.73
	100			242.68	0.80				
	50			19.50	1.32				
	LOQ			25.16	2.65				
Impurity B	150			362.85	0.63	667	793	1.74	2.07
	100			283.54	0.74				
	50			482.68	2.38				
	LOQ			77.51	3.22				
Impurity C	150			151.13	0.377	502	437	1.88	1.65
	100			213.62	0.810				
	50			202.50	1.55				
	LOQ			74.55	3.77				

Table 4 Stability and Robustness Data for Proposed Method of 3MBCA and Impurity A, B, C

Substance	Stability At RT for 24 hrs and 48 hrs of Area (LOQ Level)		Stability At 5°C for 24 hrs and 48 hrs of Area (LOQ Level)		Robustness Mobile Phase PH of 100 % level of Area		Robustness Flow rate of 100 % level of Area of $t_R$different	
	SD	RSD	SD	RSD	3.3 PH RSD	3.5 PH RSD	1.0/ml different of t_R	0.8/ml different of t_R
3MBCA	28.28	3.07	14.14	1.51	0.67	0.34	0.31	0.52
Impurity A	21.21	2.34	10.60	1.15	1.15	0.87	0.35	0.51
Impurity B	15	0.62	10	0.41	0.22	0.42	0.59	0.25
Impurity C	45	2.32	15	0.76	0.44	0.76	0.34	0.53

Although no significant degradation of 3MBCA and three impurity produced figure receptivity at a very small rate capable of elevating their concentration. Representative linearity and range results in 3MBCA are included in table 4.

Robustness

The robustness of the method was evaluated by analyzing standards and best solutions at the methanol nominal concentration of 3MBCA (≈100% level) in the presence of 3MBCA and Three impurity at 0.5 % of the parent drug peak Figure. The parameters altered were the flow rate (±10%) 0.8,1.0mL/min instead of 0.9 mL/min, pH(±0.1)3.3, 3.5 instead of 3.4 and the different column. Representative linearity and range results in 3MBCA Bulk drug intermediate are included in the result were summarized in table 4.

CONCLUDING REMARKS

In conclusion a process related impurity of impurity A, B, C to synthetic of 3 MBCA route. The prepared impurity standard was used during analytical method validation studies. This work also supported the optimization stage of the process development and enabled us to see and control the critical points of the process. The knowledge of the impurity A, B, C helped us to control the amount of impurities by a newly developed validated HPLC method was as per ICH guidelines. The Reversed-Phase high performance liquid chromatography method stationary phase with binary gradient mode rapid, accurate, simple and selective for the simultaneous determination of 3MBCA and its three process related impurity A, B, C in the bulk drug intermediate and synthesis drug intermediate online observed was fully validated and proved to be reliable sensitive, precise and robust a can be useful for routine analysis and quality control of 3MBCA and impurities in pharmaceutical industry.

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