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LC-MS METHOD DEVELOPMENT AND VALIDATION FOR QUANTIFICATION OF CROTAMITON AND ITS IMPURITY (A) IN MARKETED DOSAGE FORMS

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ABSTRACT

An efficient and convenient LC-MS (reverse-phase high-performance liquid chromatography coupled with mass spectroscopy) method has been developed and validated for quantification of Crotamiton (CTM) and its impurity A (CTMI A) in the marketed dosage form. The method was intended for separation of CTM and its impurity CTMI A, conditions are aimed at sensitive determination of CTMI A, as it is existing in very low concentration in its API (CTM). Chromatographic separation was achieved on Knauer C18 vertex plus column (100 X 4.6 mm) using methanol: Acetonitrile: 1.0 N ammonium acetate (55:44:1) as a mobile phase in isocratic mode with UV detection at 264 nm. Quantification was achieved using an electrospray ion interface operating in positive mode, under multiple reaction monitoring conditions.

Keywords: RP-HPLC, Mass Spectroscopy, Crotamiton, Method Development, Validation.
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INTRODUCTION

Crotamiton (CTM) belongs to organic heterocyclic compounds called anilides. The drug is scabicidal (for treating scabies) and antipruritic agent (anti-itching agent) available as a cream or lotion for topical use only, prescribes for skin infections caused by mites. The mechanism of CTM activity on mites that by inciting TRPV4 (transient receptor potential vanilloid 4) channel that is expressed in the skin, primary sensory neurons. It is also used for the treatment of sunburn. Impurities may influence the safety of pharmaceutical products. It is mandatory to know the structural details of impurities that exceed 0.1% in the bulk drugs according to the ICH.[1,2] Profiling of these impurities is an important part of the manufacturing process and regulatory expectation of the pharmaceutical products. To build a high degree of purity in drug substances and drug products, it is required to carry out all the investigation for standards of drugs and impurities. Among all other techniques, liquid chromatography coupled with mass spectroscopy is one of the powerful tools for the identification of impurities and drug metabolites. This technique offers selectivity and specificity at extremely low concentrations and is steadily applied to scrutinize impurity during pharmaceutical product development and manufacturing process.

The present work is aimed to develop an LC-MS method for the determination of CTM and its impurity A (Crotamiton impurity A) in bulk and marketed dosage form. Chemically CTM (Fig.-1) is (E)-N-ethyl-N-(2-methylphenyl) but-2-enamide having molecular formula $C_{13}H_{17}NO$ with molecular mass 203.28 g/mol. Crotamiton impurity A (CTMI A) (Fig.-2) is structurally similar to the CTM, it is N-ethyl-N-(2-methylphenyl) but-3-enamide.

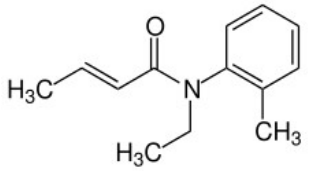

Fig.-1: Chemical Structure of Crotamiton (CTM)

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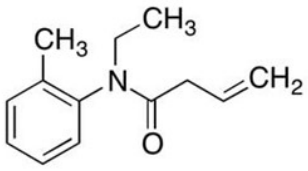

Fig.-2: Chemical Structure of Crotamiton Impurity A (CTMI A)

EXPERIMENTAL

Instrumentation

An HPLC (Agilent Technologies) equipped with quaternary G1311 pump, COLCOM G1316A thermostat column temperature control, thermostatic autosampler G 1329A with a sample volume of 0. 1 – 1500 µL and variable programmable UV detector G 1314 A is used for separation. The LC is operated and integrated with Agilent chemstation LC software. Waters ZQ mass detector (Model LAA 1369) with waters empowers system used for molecular mass analysis. The mass spectra were taken in ESI (Turbo Ion Spray) positive mode within mass range of 40-1000 a.m.u. and analyzed in the Quadra pole analyzer with MS tune of 3.0 kv (capillary), 40 V cone, # V extractor at a source temperature of 300^0C.

Chemicals

All chemicals used in this study like ammonium phosphate, potassium di hydrogen phosphate, ammonium acetate were analytical grade from Merck life sciences pvt. ltd, Bengaluru. HPLC grade acetonitrile, water and methanol from Thermo Fisher Scientific India private limited, Mumbai. CTM reference drug standard and CTMI A impurity were procured from a reputed organization.

Preparation of Stock and Working Solutions

Accurately weighed 10 mg of CTM and CTMI A were dissolved individually in 10 ml of methanol to prepare 1 mg/ml (1000 µg/ml) stock solutions. Both solutions were filtered through the 0.45 µm Millipore filter paper. A series of CTM standard working solutions were prepared by diluting the stock solution with methanol to obtain the desired concentration. Similarly, impurity working dilutions are prepared from its stock solution.

Sample Solution

The marketed formulation of CTM (Crotorax) cream manufactured by Abbott Healthcare Pvt ltd, Mumbai was purchased from a local pharmacy. About 100 mg of CTM cream was accurately weighed and transferred into a volumetric flask and dissolved with methanol. The solution was sonicated for fifteen minutes and filtered through the 0.45µm Millipore filter paper. It was further diluted with methanol to prepare 1000 µg/ml of sample stock solution.

Method Development

Initial method conditions like mobile phase, column and detector conditions were optimized primarily. As the drug CTM and its impurity CTMI A are freely soluble in methanol, different solvents with different ratios along with methanol were studied as mobile phase. Different stationary phases (C8 and C18) and columns with different brands (zorbax, Thermo, Waters, Zodiac**,** Knauer) were evaluated to obtain a symmetric peak with better resolution. The ultraviolet detector wavelength was also optimized to obtain a good peak response. Among all studied mobile phases methanol with acetonitrile in the ratio of 55:44 was found suitable for separation of both CTM and its impurity with good resolution. The pH of the mobile phase is increased by the addition of 1ml of 1.0 N ammonium acetate. Knauer C18 vertex plus column with 100 × 4.6 mm dimensions and packing material Eurospher II 100-5 was effectively separated both compounds with good resolution among all studied columns. All other method parameters were also optimized to achieve peaks with system suitable conditions with sensitive detection. Mass spectroscopy conditions like ion spray voltage and source temperatures were optimized to achieve the molecular mass data at lower concentrations. Nitrogen gas as desolvation flow was maintained between 10-20 µl/min.

Electrospray ionization in positive MRM mode was used for quantification of CTM and TMI A respectively.

Method Validation

After obtaining satisfactory separation of CTM and CTMI A with good peak response and resolution, the optimized conditions are validated according to the ICH guidelines. To demonstrate the feasibility of the proposed method, validation was performed about specificity, linearity, precision, accuracy, ruggedness, robustness, recovery, the limit of quantification (LOQ), the limit of detection (LOD) and solution stability. The specificity of the method was established through the study of resolution factors of the TM and CTM A impurity peak. The linearity was performed by diluting the drug and impurity stock solution to the required concentrations. The solutions containing six different concentrations between 40-140 µg/ml of CTM and 4-14 µg/ml of CTMI A were used for linear regression analysis. The calibration curves were drawn by plotting the peak area corresponding to its concentration. The slope and intercept of the calibration curve were calculated. System precision was established by injecting a standard CTM at 80 µg/ml and CTMI A at 8 µg/ml test concentrations.

The precision was evaluated by injecting six individual injections at intraday and interday analysis to determining the percent relative standard deviation (%RSD). The ruggedness of the method also determined by injecting six individuals of standard CTM at 80 µg/ml and CTMI A at 8 µg/ml test concentrations by a change in the analysis and %RSD was measured. The Recovery (accuracy) of the proposed method was evaluated in spiked samples were studied at three different concentration levels (50, 100 and150%). The robustness of the proposed method was also evaluated by calculating the percentage recovery of the standard concentration injected at the change in mobile phase composition, temperature and detector wavelength. The stability of the sample solution of the impurities was evaluated by analyzing the sample solution at different time intervals at room temperature. The percent of recovery was calculated to measure the solution stability. The LOD and LOQ of the proposed method were determined by considering the standard deviation of the response (s) and the slope of the calibration curve (m) values using the formula (LOD=3.3 s/m) and LOQ was calculated from the obtained LOD values (LOQ= 3.3 X LOD).

RESULTS AND DISCUSSION

After several trials of LC conditions, we observed that, the Knauer C18 vertex plus column (100 X 4.6 mm) and isocratic (UV detection at 264 nm.) elution of methanol: acetonitrile: 1.0 N ammonium acetate (55:44:1) as a mobile phase with 1ml/min. the flow rate was suitable for successful separation of both CTM and CTMI A at 5.76 min. and 3.533 min. respectively with a resolution of 4.2. Nitrogen gas as desolvation flow maintained between 10-20 µl/min. and electrospray ionization in positive multiple reaction monitoring modes was suitable for quantification of CTM and CTMI A respectively. The API CTM and its impurity were well separated from each other and are presented in Fig.-3.

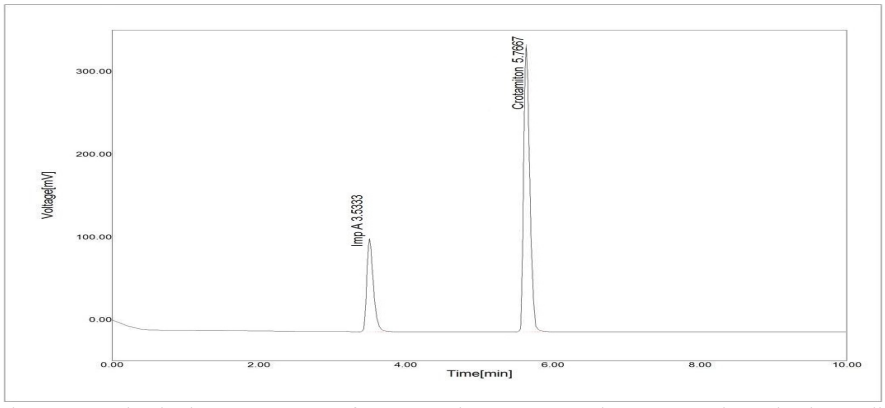

Fig.-3: Standard Chromatogram of CTM and CTMI A under proposed Method Conditions

Identification is based on the comparison of the retained characteristics, simply the retention time. The peaks in the mixed solutions were identified by injecting the CTM and CTMI A individually. The role of

MS is the fact that the mass spectra of compounds are specific to allow their identification with a high degree of confidence. The molecular mass of both peaks is found similar to their original mass values which are presented in Fig.-4 and 5.

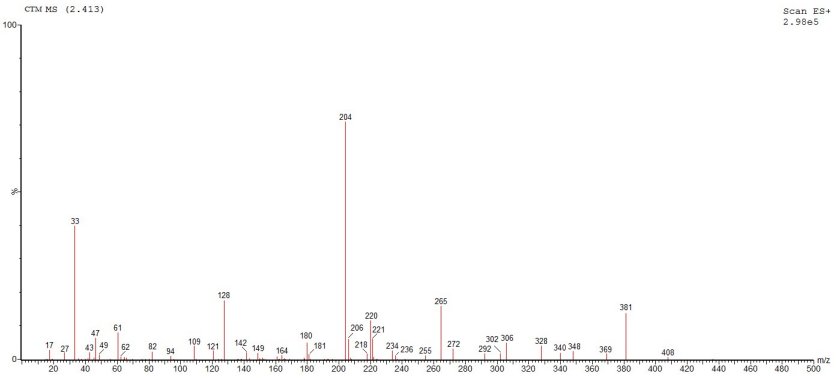

Fig.-4: Mass Spectra of CTM at Positive Electrospray Ionization (ESI) Mode

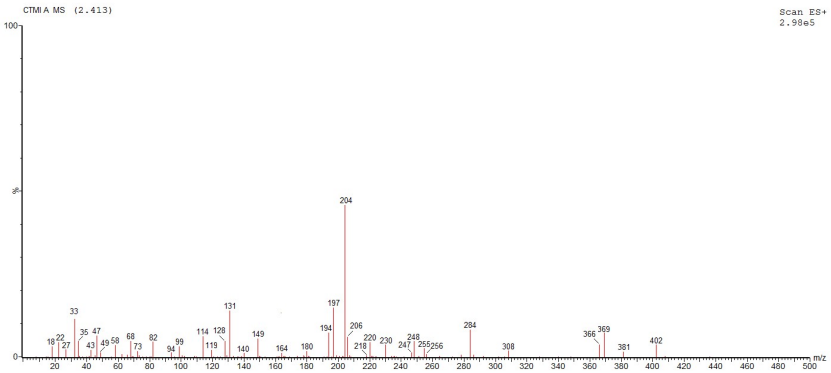

Fig.-5: Mass Spectra of CTMI A at Positive Electrospray Ionization (ESI) Mode

Therefore, the capillary of LC separates impurity with a high degree of purity using the mass spectrophotometer is meant for specific identification. The linearity of CTM and CTMI A was satisfactorily demonstrated with a six-point calibration graph from 40-140 µg/ml for CTM and 4-14 µg/ml for CTMI A respectively. The slope, intercept and correlation coefficient values are derived from the linear least squares regression analysis and are presented in Table-1. and Fig.-6 and 7.

Table-1: Results of Linearity Study

S. No.	CTM		CTM I A	
	Concentration in µg/ml	Area	Concentration in µg/ml	Area
1.	40	97127.3	4	18012.3
2.	60	134127	6	24362.5
3.	80	165488	8	35556.7
4.	100	198108	10	49105.1
5.	120	235448	12	58749.2
6.	140	271365	14	66807.3
	R^2 = 0.9992 Slope = 1725.4 Intercept = 28326		R^2 = 0.9915 Slope = 5152.6 Intercept = - 4274.7	

The precision and ruggedness of the proposed method were evaluated and checked by calculating the % RSD of six replicate determinations by injecting standard solutions containing 8 µg/ml of CTMI A and 80 µg/ml of CTM and results are presented in Table 2. The recovery of the proposed method was evaluated using the standard addition method by spiking the drug and its impurity concentration separately to the standard solution of 4 µg/ml of CTMI A and 40 µg/ml of CTM at 50%, 100% and 150% levels and results are presented in Table-3.

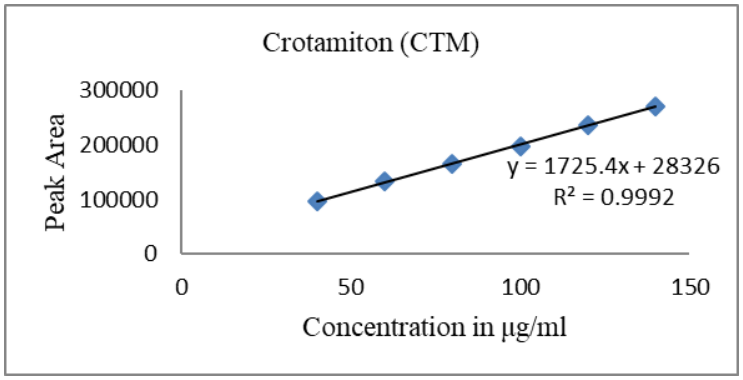

Fig.-6: Calibration Curve of CTM

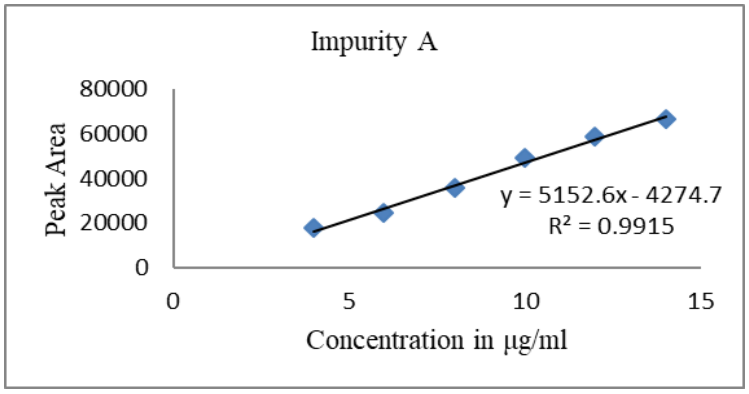

Fig.-7: Calibration Curve of CTM Impurity A

Table 2: Summary of Method Validation

S.No.	Validation Parameter	Result	
		CTMI A	CTM
1.	Linearity range	4-14 µg/ml	40-140 µg/ml
2.	Intraday Precision	1.63	0.72
3.	Interday Precision	1.65	0.82
4.	Ruggedness	1.77	1.16
5.	Robustness	98.1-101.15	98.9-101.1
6.	LOD	1 µg/ml	10 µg/ml
7.	LOQ	4 µg/ml	40 µg/ml
8.	Recovery	98.4-101.73	98.0-101.8

Table-3: Results of Recovery Study

S.No.	Recovery	CTMI A Area	Recovery in %	CTM area	Recovery in %
1	50%	24336.5	99.89	131448.6	98.00
		24784.3	101.73	133699.5	99.68
		24153.6	99.14	135487.2	101.01
2	100%	35002.3	98.44	166394.5	100.55
		35561.2	100.01	167530.2	101.23
		35394.8	99.54	163593.7	98.99
3	150%	48663.5	99.10	201677.1	101.80
		48416.2	98.50	197990.6	99.94
		49228.1	100.20	196787.1	99.33

The robustness of the method also evaluated by calculating the % of recovery of chromatograms achieved at deliberate modification of the proposed conditions in the flow rate (0.9, 1.1 ml/min.) of mobile phase and temperature (28^0C, 32^0C) of the column and pH of the mobile phase (5.2, 5.4). All the recoveries achieved are within the specification limit and results were presented in table 2. LOQ of the method was

found lowest calibration concentration i.e 4 µg/ml for CTMI A and 40 µg/ml for CTM. The LOD under the proposed method was found to be 1 µg/ml for CTMI A and 10 µg/ml for CTM respectively. The stability of CTM drug and its impurity A in methanol solution was evaluated by keeping them in an autosampler and observing the variations in their peak area. From the stability results, it was found that the drug CTM and its impurity A are stable in diluents for more than 24h.

Analysis of marketed formulation by the proposed method was applied to Crotorax cream (20 grams containing 10% of dosage) and assay results (100.7%) were found to be within the acceptable limit. The method successfully detected the impurity presented in the formulation also and the specificity of the method was also observed by that the common excipients in the formulation cream did not interfere at the retention times of CTM and CTMI A. So the proposed method is a novel LC-MS one for the determination of CTM and CTMI A, where other methods described by different authors are intended for various applications. [3-5]

CONCLUSION

The LC-MS method developed for separation and quantification of CTM and CTMI A in standard and marketed dosage form has been validated and found specific, accurate and precise with satisfactory results. The LOD and LOQ of the method were found very low for impurity that can easily be identified at very low concentration. The developed method can be conveniently used for determining the quality of CTM in bulk drugs and also successfully applied to the formulation to achieve satisfactory results.

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