Development and Validation of Three Potential Genotoxic Impurities by Liquid Chromatography Single Quad Mass Detector in Iomeprol

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

A liquid chromatography with single quadrupole mass detection method was developed for the determination of potential genotoxic impurities (PGIs) in the Iomeprol active pharmaceutical ingredient. Chromatographic separation was achieved on an Agilent Eclipse plus C8 column (100 mm x 2.1 mm x 1.8 μm) with 0.1% formic acid in water as mobile phase A and acetonitrile as mobile phase B in gradient elution mode at a 0.1 mL/min. Executed validation summary demonstrated that the mass detection method had highly sensitive and selective. A linear calibration curve (correlation coefficient, r> 0.999) was attained at the concentration range of 0.1-125 ppm for three PGI's. The Limit of Detection of Imp-A, Imp-B and Imp-C in drug substance of Iomeprol is 0.05 ppm. The accuracy was confirmed by calculated recoveries (98.4-101.5%). The precision was tested at three levels: injection repeatability, analysis repeatability and intermediate precision. The calculated relative standard deviations were within the specification. The developed method was able to quantitate all three PGI's at a concentration level of 1 μg/mL.

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1. INTRODUCTION

These Genotoxic impurities have ability to react with Deoxyribonucleic acid (DNA), causing oncological risk in patients even at lower level (1 ppm below). Typically, these impurities are produced during the manufacturing of drug substance and other possible source is synthesis of raw materials, intermediates, by-products and degradation of drug substances in storage. Such type of chemicals may show unwanted toxicity i.e., Genotoxicity [1,2] or carcinogenicity. As result of genotoxicity, the presence of these impurities in drug substance has become an increasing concern of various regulatory authorities, pharmaceutical companies and medical consultants [3]. According to ICH guidelines, these impurities should not be exceeding the reported threshold or should not be raise during its synthesis or storage. These Genotoxic impurities may or not actually be present in the API but are identified as the ones that can theoretically arise during manufacture or storage [4]. Thus, toxicity nature of impurities is identified either by published data on the chemical structure along with the demonstration of the Genotoxic nature of impurity or by its alerting functional structure [5]. The impurities which have structural alerts and may cause the cancer in patients are called as potential Genotoxic impurities (PGIs) [6]. Iomeprol is classified as a water soluble neurotrophic low osmolar X-ray contrast medium. It is sold under the trade name Imeron and it comprises with three iodine atoms around aromatic ring and shows equivalent diagnostic efficiency and a similar adverse event profile to that of other non-ionic contrast media such that Iohexol, Iopamidol, Iotrolan, Iodixanol et,. Iomeprol synthesized by using the different types of intermediates and reagents. While a few intermediates were considered to be Genotoxic impurities as they have structural alerts on CASE ultra-software and those shown in Fig 1.

As per the TTC approach the Genotoxic impurities in the drug substance [6,7] should be 1.0 ppm as a daily dosage of 1.5 g/day. So, these impurities should be limited to an acceptable level of 1.0 ppm by conventional process such as fractional crystallization andrecrystallization and the amount of impurities in Iomeprol API should checked often its preparation. Development of a method for the determination of Genotoxic impuritiesat lower level using ultraviolet-visible spectrometry, gas chromatography and high-performance liquid chromatography is great challenge in the pharmaceutical industry as the sensitivity of these instruments is low [8-12]. To the best of our knowledge, no reports have been available for the detection very low impurities in Iomeprol API. As per the literature still now ICMS's are tested in plasma and in water [13-17] Thus, in continuation of our work on the development and sensitive LCMS method was developed for the determination of potential Genotoxic impurities materials: Imp-A, Imp-B and Imp-C in Iomeprol drug substance and the validation of this method was performed according to the ICH guidelines [18].
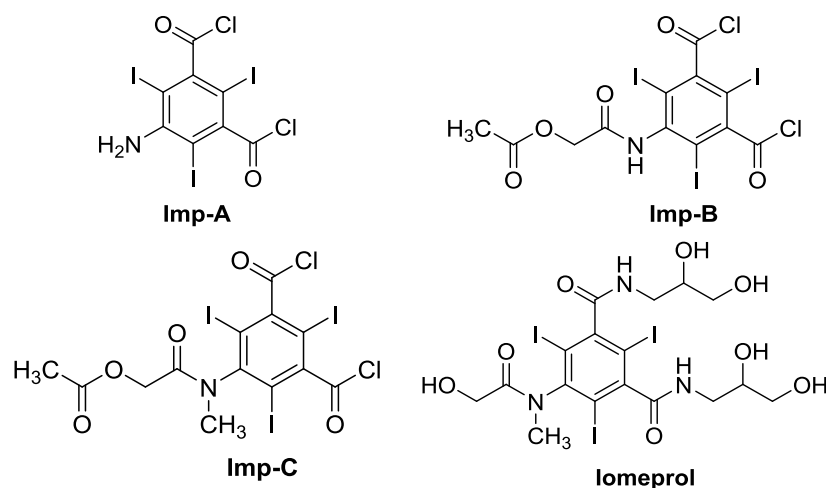

Fig. 1. Structure of Iomeprol and three genotoxic impurities

2. MATERIALS AND METHODS

2.1 Materials

Iomeprol active pharmaceutical ingredient, Imp-A, Imp-B and Imp-C were received from Sigma Aldrich, India. The following HPLC grade solvents of Acetonitrile were purchased from J.T. Baker. Analytical grade reagent of Formic acid was purchased from Merck. LC-MS grade water purchased from Fisher scientific.

2.2 Instrumentation and Method Conditions

Liquid chromatography mass spectrometer consists of Agilent 1290 infinity-I pump at flow rate of 1.0 ml/min an Agilent auto sampler an Agilent 300 UV detector set at 270 nm and a Agilent 6130 single quadruple mass spectrometer equipped with an Electrospray ionization mode and operating with Chemstation software were used. An Agilent HPLC separation column of Agilent Eclipse plus C8 (USP L7) dimensions of 100 mm x 2.1 mm and 1.8 μm were used at 40ºC column temperature. Gradient program was used mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in Acetonitrile), injection volume is 10 μl and the run time is 15 minutes. After emerging of the desiredanalytes, later eluting peaks are washed out of the column with high organic solvent medium.A single quad MS equipped with a negative electro spray ionization source was used in the SIM mode. In this method Imp-A, Band C were monitored with its molecular ion and daughter ion m/z shown in Table 1. The equipment was set with a drying gas flow, nebulizer pressure, gas temperature, and spray voltage of 11 L/min, 30 psi, 250°C, and 3500 V respectively was used for MS.

2.3 Preparation of Standard and Sample Solutions

The sample iomeprol solution was prepared at 20 mg/ml in diluent of Acetonitrile: water 20:80 ratio. Stock standards of the Imp-A, Imp-B and Imp-C were prepared at a concentration of 1 mg/ml in diluent. Subsequently standard mixture solutions containing the three impurities at a concentration of 1μg/ml (equivalent of 1ppm) in diluent were obtaining by diluting the stock standards for analysis in Iomeprol samples.

3. METHOD VALIDATIONPARAMETERS

3.1 Linearity

To demonstrate a calibration plot we prepared five solutions in the concentration range of 0.1-1.25 μg/ml. nearly 12.5 mg of each individual impurity was accurately weighed and transferred to 10 ml volumetric flask and completely dissolved in 2 ml diluent, after sonication for 2 min the rest of the volume is diluted with diluent and was used as the stock standard solution. Impurities were diluted to concentrations of 0.10, 0.50, 0.75, 1.0, 1.25 μg/ml. The correlation coefficient, intercept and slope and were determined by linear regression data analysis.

3.2 Limit of Quantification and Limit of Detection

Accurately measure the appropriate amount of 12.5 mg/ml solution under the linearity and dilute with diluent quantitatively and stepwise if necessary. The diluted solutions were separately injected into the chromatograph. LOQs and LODs were defined the concentrations that could be detected and yield signal to noise (S/N) ratios of 10:1 and 3:1 respectively.

3.3 Accuracy

To determine the Liquid chromatography mass spectroscopy method from spiked recovery experiments by using three concentration levels of Iomeprol solutions containing three impurities (20 mg/ml). The test concentrations 1.25 (125% level) 1.0 (100% level) and 0.10 (LOQ% level) were prepared and analyzed by determining the content calculated in comparison with the nominal concentration of each impurity. Accuracy was reported as percentage of mean recovery and relative standard deviations (%RSD) were calculated for each concentration level.

Table 1. Mass spectral data for three PGI's

Compound	Parent ion	Daughter ion
Imp-A	594.19, 596.36 & 598.85	479.59
Imp-B	694.46, 496.58 & 598.32	467.75
Imp-C	708.59, 709.78 & 711.53	509.11

3.4 Precision

To determine the precision for present LC-MS method, we prepared six fresh preparations of standard mixture solutions containing three impurities at a concentration of 1.0µg/ml and established each injection and analysis repeatability. Whereas, intermediate precision was tested in same manner by another analyst, another day. The precision of the method was evaluated by calculating with relative standard deviation.

3.5 Robustness and Solution Stability

The Robustness of the method was determined by various experimental conditions such as the column temperature, flow rate and source temperature of MS. To establish the solution stability, we analyzed triplicate injections of the standard mixture solutions at different time intervals such as 2, 24 and 48 hours at room temperature. The results estimated by %RSD.

4. RESULTS AND DISCUSSION

4.1 Analytical Method Development

The main aim of the LC/MS/MS method in this study was to separate and quantify three PGIs (Imp-A, Imp-B and Imp-C) in the iomeprol active pharmaceutical ingredient. Sample preparation is an important step in the three genotoxic impurities analysis to control side reaction with diluent, matrix effects, and to improve the sensitivity as well as to achieve better analyte recovery. Several columns were tested to obtain the most appropriate peak shape and separation. Among the all Agilent Eclipse plus C8 column (100 mm x 2.1 mm x 1.8µm) was found most suitable regarding both peak shape and separation, as well as baseline separation. The mobile phase was operated in gradient mode using 0.1% formic acid in water and acetonitrile (see the subsection Operating conditions of LC-MS/ MS). The gradient programme was provided in Table 2 to be more efficient in achieving optimum separation of impurities from each other with respect to drug substance peak (resolution between impurity B & C and C&A is 2.1 and 2.6 respectively). In addition, acetonitrile and methanol were tested as a potential organic phase, and acetonitrile was chosen for its much better elution efficiency. In case of methanol, we could not achieve recovery of three PGIs due to impurities were converted to corresponding ester compound sand the selection of mobile phase and columns details are summarized in Table 3. The flow rate of the mobile phase was maintained at 1.0 mL/min, with the column temperature set at 40 °C. The retention times of Imp-A, Imp-B, and Imp-C were observed to be 5.21, 3.75 and 4.32 respectively. The Mass spectrum of three PGI's are given in Figs. 2-4.

Table 2.Gradient programme

Time (min)	% Mobile phase A	% Mobile phase B
0	80	20
2	40	60
6.5	40	60
9	10	90
11	10	90

Table 3. Different trials

| Trail	Column	Buffer	Observation	Resolution
01	YMC-Triart C18 column (100 mm X 2.0 mm, 1.9 mm)	0.1% TFA in water and Acetonitrile	Poor peaks resolutionwas observed.	0.9
02	Waters Symmetry C18 (100 mm X 2.1 mm, 3.5 µm)	0.1% TFA in water and Acetonitrile	Peaks were overlapped	-
03	Agilent Eclipse plus C8 (100 mm x 2.1 mm x 1.8µm)	0.1% TFA in water and Acetonitrile	Resolution not sufficient	1.2
04	YMC-Triart C18 column (100 mm X 2.0 mm, 1.9 mm)	0.05% Formic acid in water and Acetonitrile	Poor resolution with good peak shape	1.0
05	Waters Symmetry C18 (100 mm X 2.1 mm, 3.5 µm)	0.05% Formic acid in water and Acetonitrile	Peaks were overlapped	1.1
06	Agilent Eclipse plus C8 (100 mm x 2.1 mm x 1.8µm)	0.05% Formic acid in water and Acetonitrile	Good peak shape with minimum resolution	1.4
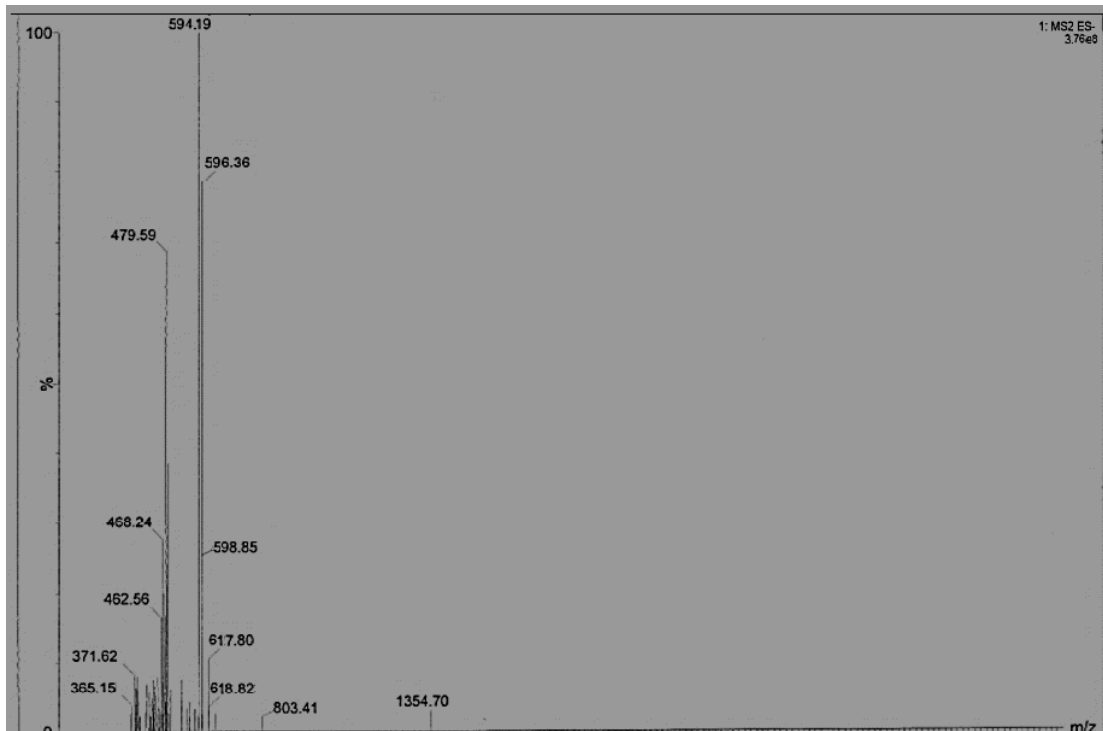
Fig. 2. Mass spectra of imp-A
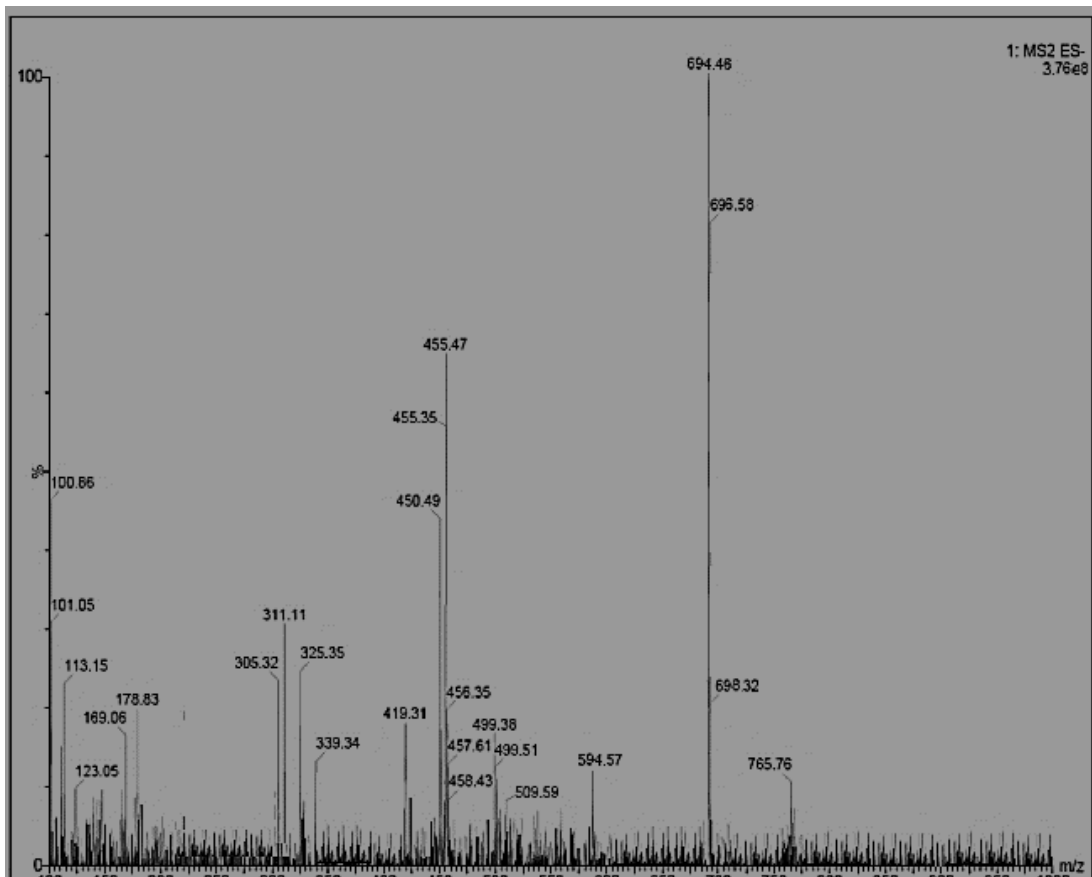
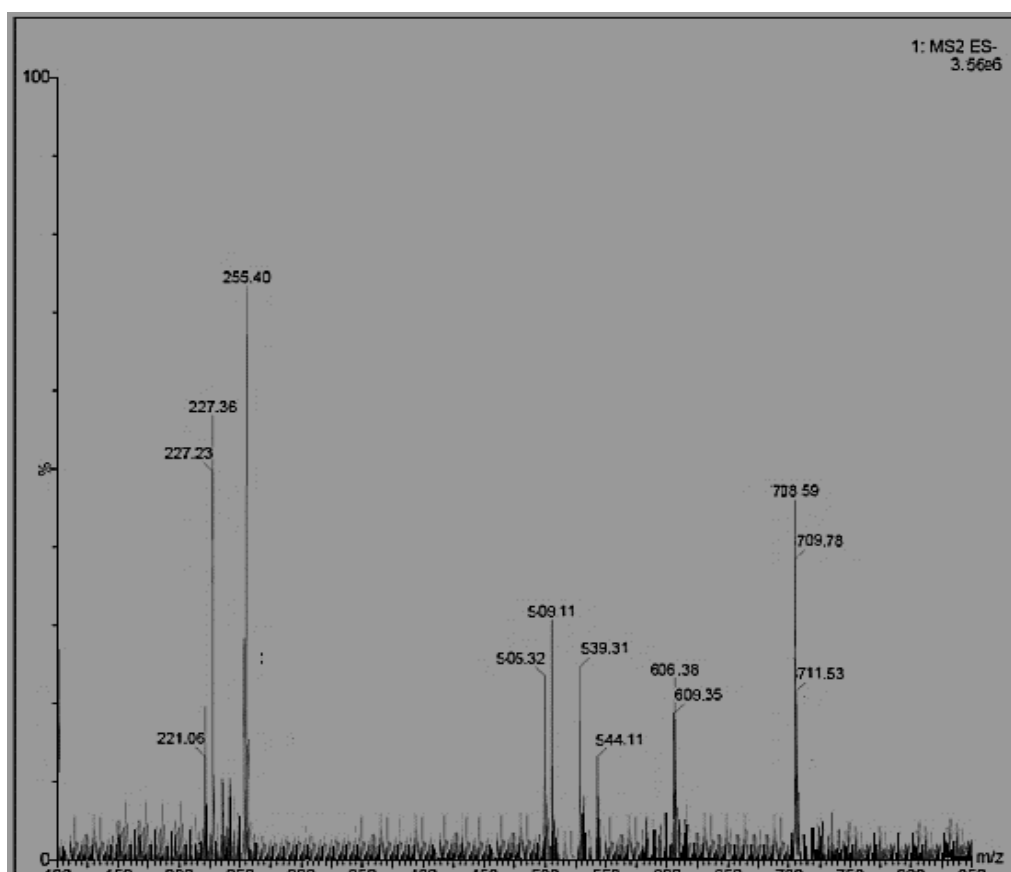
Fig. 3. Mass spectra of imp-B

Fig. 4. Mass spectra of imp-C

5. METHOD VALIDATION

5.1 Specificity

The specificity of the method was evaluated by injecting blank, individual impurities and Iomeprol at a concentration of 1.0 µg/ml. The corresponding SIM chromatograms of Iomeprol sample at impurities eluted region and LOD level impurities spiked in Iomeprol sample are shown in Figs. 5 & 6. The chromatograms show that the developed method could successfully separate the impurities from one another and from the main drug with no interference of blank chromatogram.
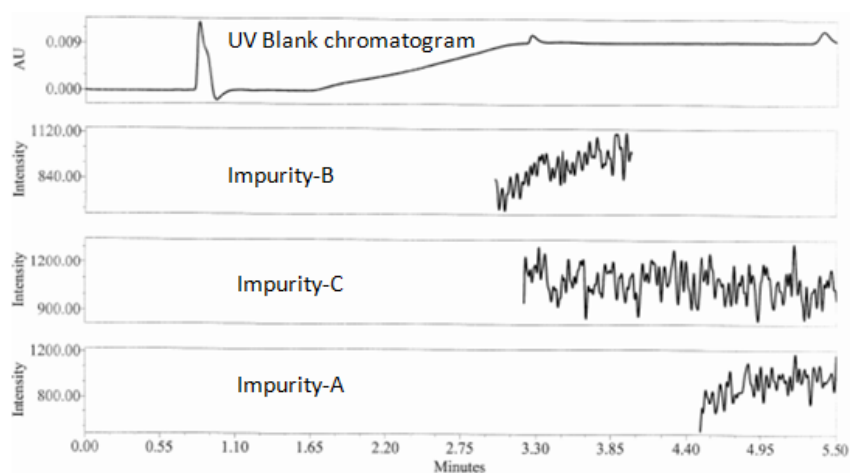

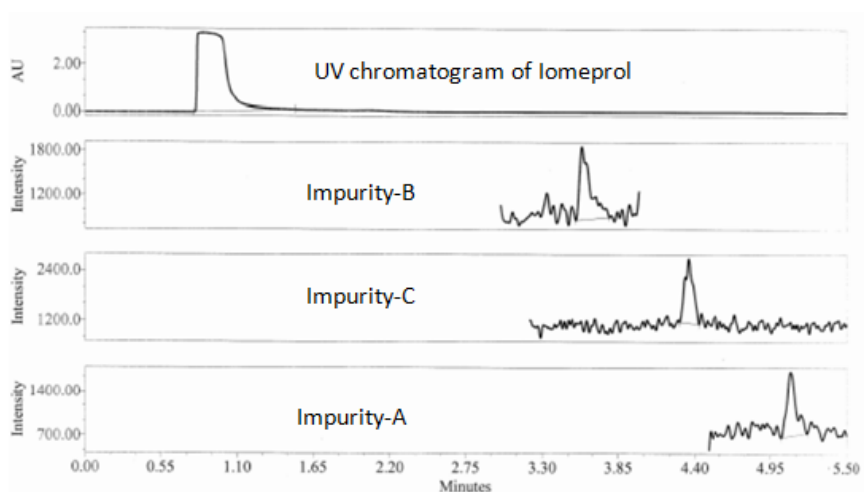**Fig. 5. Blank chromatogram of imp-A, imp-B and imp-C**

Fig. 6. LOD chromatograms of impurity-A and impurity-B and Impurity-C with sample UV chromatogram of Iomeprol

5.2 Linearity

The linearity of the method was evaluated at five different concentration levels for each impurity and shown in Figs. 7-9. This linearity was satisfactorily illustrated by using a five-point calibration graph by taking the areas in Y-axis and concentration on X-axis Figs. 10-12. Calculated the correlation coefficient as ≥0.99 and also calculated slope and intercept. Results are tabulated in Table 4.
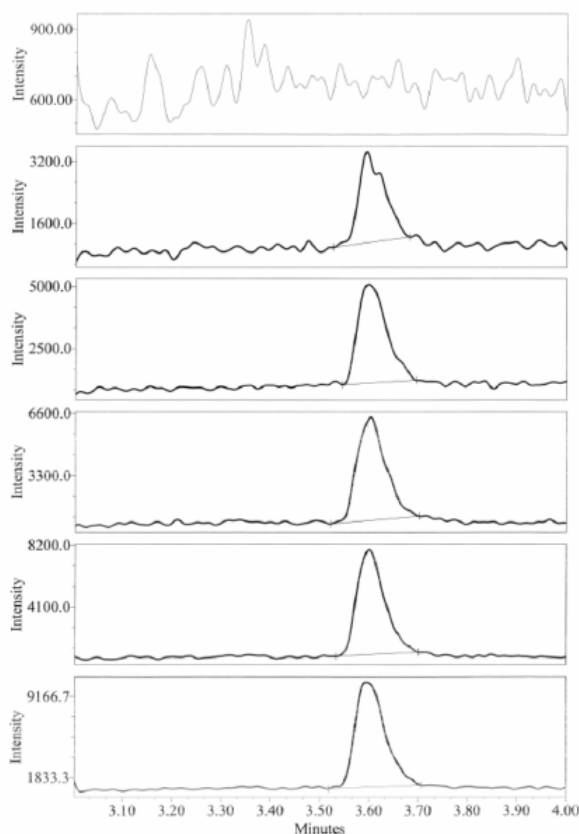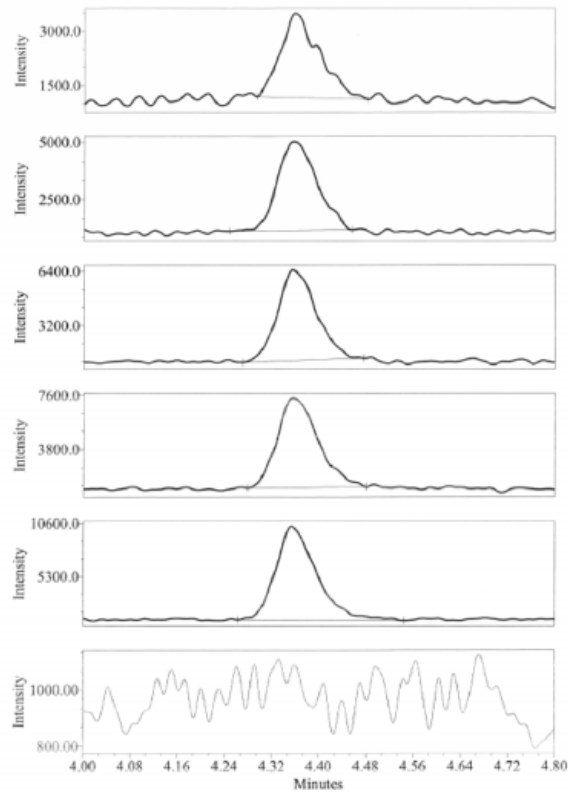

Fig. 7. Linearity chromatograms of imp-A
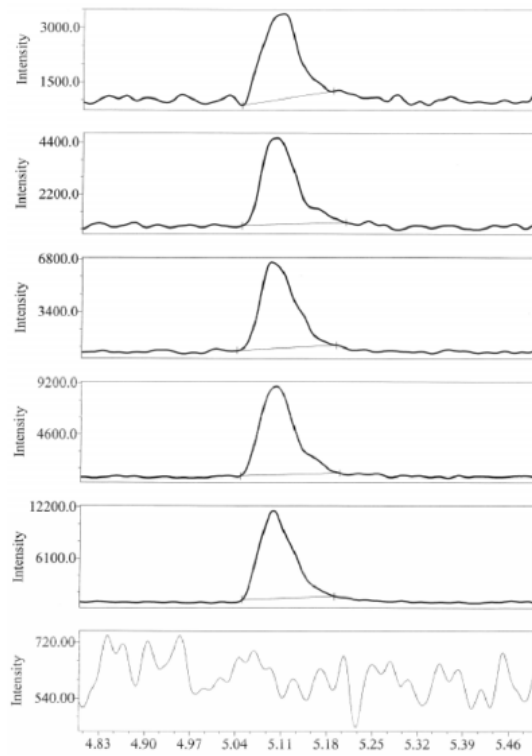
Fig. 8. Linearity chromatograms of imp-B

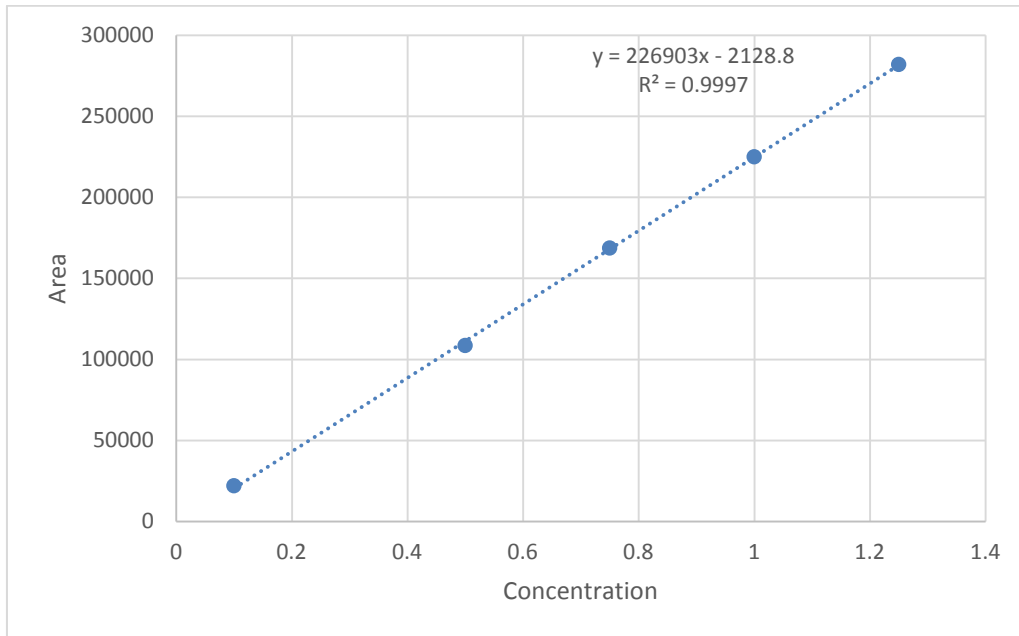**Fig. 9. Linearity chromatograms of imp-C**

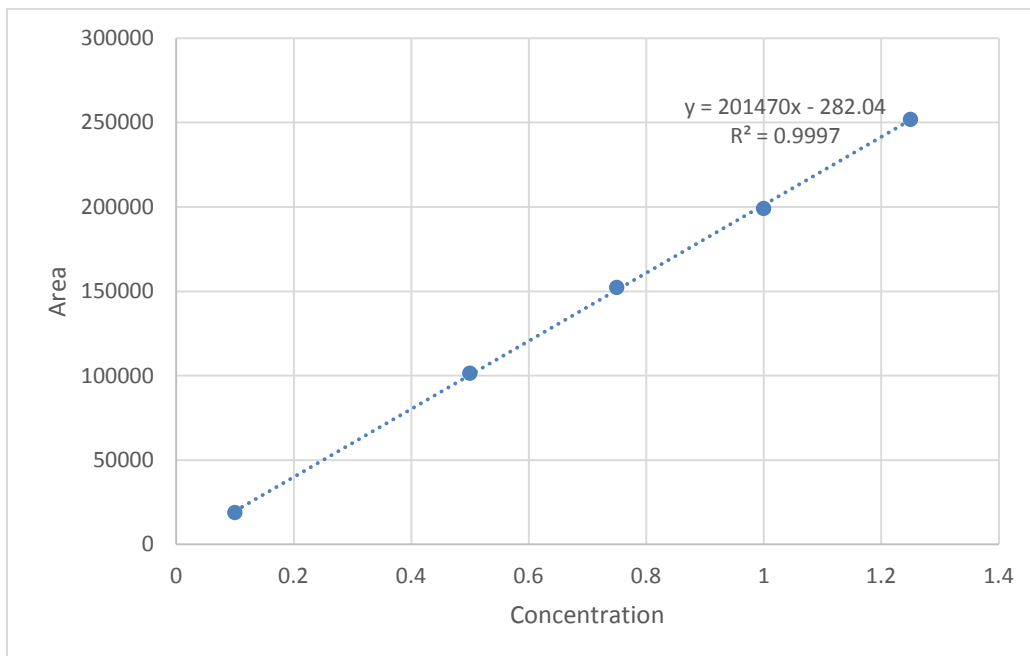**Fig. 10. Linearity plot of imp-A in the concentration range of 0.1–1.25 ppm**

Fig. 11. Linearity plot of imp-B in the concentration range of 0.1–1.25 ppm

Table 4. Linearity of imp-A, imp-B, imp-C and imp-D

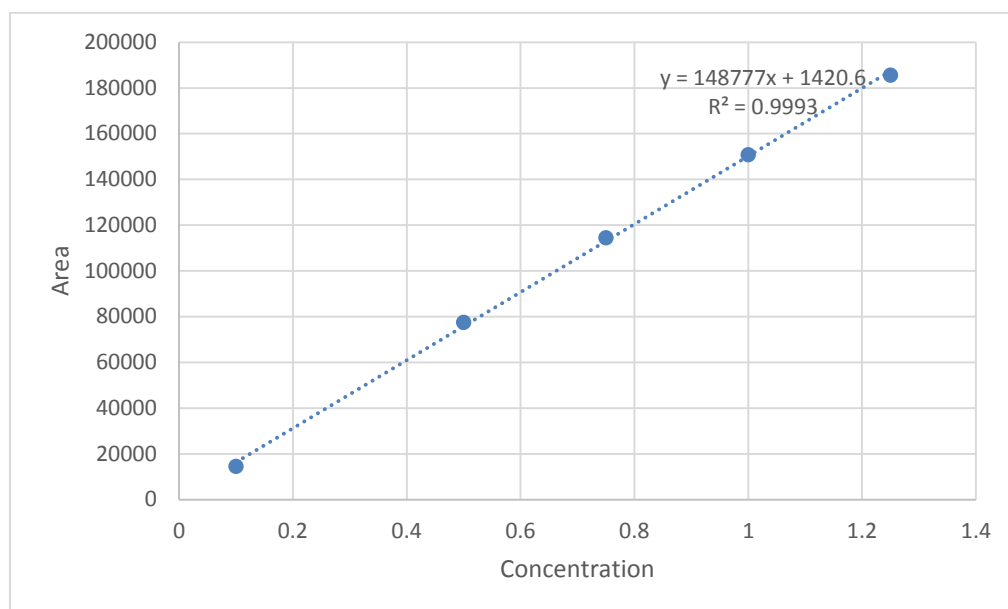| Level	Imp-A area	Imp-B area	Imp-C area
0.1	22037	18956	14477
0.5	108566	101505	77453
0.75	168695	152359	114447
1	224963	199135	150755
1.25	281945	251926	185567
Correlation coefficient	0.9997	0.9997	0.9993

Fig. 12. Linearity plot of imp-C in the concentration range of 0.1–1.25 ppm

5.3 Accuracy

The accuracy of the method was evaluated through spiked recovery experiments. Authentic impurities were spiked into 20 mg/ml Iomeprol in triplicate using concentration levels of LOQ (0.10µg/ml), 100% (1.0µg/ml) and 150% (1.25µg/ml). Good recoveries in the range of 95.41-102.62% with RSD% values below 1.5 were achieved as shown in Table 5.

5.4 Precision

Precision was examined by injecting six individual preparations of the standard mixture

solution containing impurities at the limit level (1.0µg/ml). The method validation results summarized in Table 5 indicate that our established method can reliably quantify these impurities in Iomeprol.

5.5 Robustness and Solution Stability

Robustness of the method was studied by changing experimental conditions such as the flow rate, column temperature and source temperature of the MS. Flow rate of the mobile phase 1.0 ml/min, performance of the method was studied at floe rates of 0.8 and 1.2 ml/min. the effect of column and source temperature was

Table 5. Summary of the validation report of the liquid chromatography mass spectrometry method

Parameter	Impurity-A	Impurity-B	Impurity-C
Detection limit (µg/ml)	0.05	0.05	0.05
Quantitation limit (µg/ml)	0.10	0.10	0.10
Precision	-	-	-
Injection repeatability% (n=6)	0.59	0.81	0.93
Analysis repeatability % (n=6)	2.46	1.96	1.87
Intermediate precision % (n=6)	2.98	1.84	1.78
Stability at room temperature (24 h) %RSD (n=3)	1.29	1.55	1.13
Stability at room temperature (48 h) %RSD (n=3)	4.98	2.53	2.25
Accuracy at LOQ level (n=3) % recovery	99.13	99.38	99.66
RSD% (n=3)	0.36	0.68	0.16
Accuracy at 100% level (n=3) % recovery	99.69	99.76	100.36
RSD% (n=3)	0.52	0.43	0.37
Accuracy at 125% level (n=3) % recovery	102.63	100.26	100.22
RSD% (n=3)	0.49	0.56	0.18

also studied at 35 and 45ºC, 350 and 450 ºC respectively. No considerable change in the chromatographic performance was noted for varied experimental conditions, which confirm the robustness of the method. The %RSD of content for each impurity was calculated the solution stability of the impurities in the sample solution was established by analyzing Triplicate of the standard mixture solutions at different time intervals (2, 24 and 48) at room temperature (Table 5).

6. CONCLUSION

A sensitive and reliable liquid chromatography mass spectrometry method was developed and validated according to USP 736 and ICH guidelines for the quantitative analysis of impurities in the API material Iomeprol. The new method was specific, precise, accurate and linear within the assessed concentration range. The detection levels of the impurities were below 0.1 µg/ml. Efficient, chromatographic separation of each impurity from Iomeprol was carried out. Using a switch valve to divert the mobile phase and eluents from the MS detector the ESI source was protected and favorable conditions were provided for analysis. As a versatile and convenient technique, the proposed method is expected to be used in evaluations of the stability of Iomeprol production and analysis of impurities as model. The LC-MS method is indispensable to producers of contrast media as it can ensure low amounts of impurities in the final API. Therefore, the results of this study will help ensure the safe use of APIs during clinical treatment.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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