# Development and Validation of an Inductively Coupled Plasma Mass Spectrometry (ICP-MS) Method for the Determination of 17 Trace Metals in Ingenol Mebutate (API)

Sanjeeva Reddy Kallam[1], Dr. J.Srikanth[2], Dr.K.Vanitha Prakash[3]

*[1]Dr. Reddy's Laboratories Ltd., IDA, Bollaram, Hyderabad, Telangana, India.*
*[2]MSN Labs Pvt. Ltd., Hyderabad, Telangana, India.*
*[3]S. S. J. College of Pharmacy, Hyderabad, Telangana, India*

***Abstract:*** In this study elemental impurities method of quantitative analysis for the determination of trace metals in Ingenol mebutate (API) by ICP-MS was validated and applied. ICP-MS is a multi-element technique characterized by high selectivity, sensitivity and detection limits much lower than other multi-element techniques. Inductively coupled plasma mass spectrometry (ICP-MS) equipped with microwave digestion is considered an excellent tool for detailed characterization of the elementary composition of many samples. In this study elemental impurities method of quantitative analysis for the determination of toxic metals (As, Cd, Ni, Hg, Pb) and other trace metals Ir, Pd, Pt, Rh, Ru, Os, Mo, V, Cu, Sn, Sb, Bi in Ingenol mebutate (API) using Indium as an internal standard by ICP-MS was validated. Several parameters have been taken into account and evaluated for the validation of method, namely: linearity, the minimum detection limit, the limit of quantification, accuracy and uncertainty. The results obtained for the recovery rates of all (17) metals between 75% and 124 % were found. The detection limits of all elements studied showed the suitability of the procedure for routine analyses. Summarizing it can be concluded that the described analytical procedures to measure the mass fractions of 17 elements in Ingenol mebutate (API) samples with established traceability and evaluated uncertainty allow to obtain reliable and internationally comparable results

***Keywords:*** *ICP-MS, Microwave digestion, Multi element analysis, method validation.*

## I.    Introduction

Ingenol mebutate is an API and actinic keratoses are premalignant lesions commonly encountered in dermatology, with risk factors that include fair skin types, age, and a history of chronic sun exposure. Cryotherapy is the most widely utilized treatment, but it is associated with the risk of scarring. Topical therapies, such as 5-fluorouracil or imiquimod, are disadvantageous for other reasons, including the longer duration of treatment and the risk of localized skin reactions with prolonged application, both of which may negatively impact patient adherence to treatment. Recently, the medical community has focused its attention on a new treatment for actinic keratoses called Ingenol mebutate. This medication is derived from the sap of the Euphorbia peplus plant, also known as petty spurge, radium weed, or milkweed. The sap is a white, sticky irritant that has long been used in traditional medicine for treatment of warts, corns, and nonmelanoma skin cancers. An Australian survey from 1986 regarding the use of home remedies for skin cancers and actinic keratoses described support among respondents regarding the effectiveness of the sap of Euphorbia peplus. In January 2012, Ingenol mebutate gel attained FDA approval for the treatment of actinic keratoses.

The purpose of this research article is validation of elemental method for determination of trace metals in Ingenol mebutate (represent the availability of seventeen elements in the API) by inductively coupled plasma mass spectroscopy (ICP-MS) method. Several parameters have been taken into account and evaluated for the validation of method, namely: linearity, the minimum detection limit, the limit of quantification, accuracy and uncertainty.The content of these elements can provide essential information for consumers, which is why the estimation of quality parameters is so important. In recent years, concentration patterns of trace elements were widely used in food authenticity studies.

Quality of measurements plays a very important role in many fields of our life, for instance in medicine, food Analysis, environmental studies or in the exchange of goods and services. Analytical chemistry aspires to obtain the most reliable analytical results, which must reflect unambiguous, true and clear values of the sample composition and to this end applies sophisticated instrumental techniques.We developed and applied analytical methods to measure the content of 17 major and trace elements in API (Ingenol mebutate)

# II. Experimental

## 2.1 Materials and reagents

The reference samples of Ingenol mebutate, provided as gift samples from Dr.Reddys laboratories Ltd. All chemicals were of analytical grade (Fisher grade) Nitric acid, Hydrochloric acid and all other reagents (Cd, Pb, As ,Hg ,Ir, Pd, Pt, Rh, Ru ,Os, Mo, Ni, V, Cu, Sn, Sb, Bi ,In were obtained from Merck chemical division, Mumbai. All solutions were prepared with double deionized water obtained by passing distilled water through a Millipore Milli-Q water purification system (Waters Corporation, Milford, MA, USA).

## 2.2 Instrument and Operating Conditions

The Inductively coupled plasma mass spectrometry (ICP-MS) model: Nexion 300x system was equipped with data acquision and processing software "Nexion used for the method development and validation from Perkin Elmer, USA. Microwave digester from PerkinElmer, USA.

**Table 1-** ICP-MS operating conditions:

| Equipment | : Inductively coupled plasma-Mass spectrometer |
|---|---|
| ICP RF Power | : 1600 Watts |
| Plasma gas flow | : 18 Lt /min |
| Scan mode | : Peak hopping |
| Mode | : KED |
| **Cell gas A** | **: 3.5 ml/min** |
| Cell gas B | : 0 |
| RPa | : 0 |
| RPq | : 0.25 |
| KED Mode Axial Field Voltage | : 350 |
| No. of sweeps | : 20 |
| No. of replicates | : 3 |
| No. of readings | : 1 |

## 2.3 Standard and Sample preparations:

**Diluent:** Transfer 31 mL of Conc HNO3 and 17 mL of Conc HCl into 1000 mL beaker containing 500 mL of Milli Q water and dilute to 1000 mL with Milli Q water.

### 2.3.1 Preparation of Standard stock solutions:
### 2.3.2 Preparation of 100 ppm Standard of Ir,Os,Mo,Pt,Pd,Rh,Ru,V,Ni,Sn,Sb,Bi and Cu:
Transfer 1 ml of 1000 ppm Standard of each element in to 10 ml volumetric flask individually and make up to the volume with diluent.

### 2.3.3 Preparation of 10 ppm Cd & Hg Standard:
Transfer 100 µl of 1000 ppm Standard of Cd and Hg element in to 10 ml volumetric flask individually and make up to the volume with diluent.

### 2.3.4 Preparation of 1 ppm Pb & as Standard:
Transfer 1 ml of 1000 ppm Standard of Pb and As element in to 10 ml volumetric flask individually and make up to the volume with diluent. This is 100ppm standard solution, from this 1 ml solution transfer to 10 ml volumetric flask individually and make up to the volume with diluent. This is 10ppm standard solution, from this 1 ml solution transfer to 10 ml volumetric flask individually and make up to the volume with diluent.

### 2.3.5 Preparation of 100 ppb Indium Standard (Used as Internal Standard):
Transfer 1 ml of 1000 ppm Indium Standard in to 10 ml volumetric flask and make up with diluent (100ppm), from this solution transfer 100 µl to 10 ml volumetric flask and make up with diluent (1ppm), from this solution transfer 5 ml to 50 ml volumetric flask and make up with diluent.

### 2.3.6 Preparation of 10J Mixed Standard:
Transfer 500 µl of 100ppm standard of Ir, Os, Mo, Pt, Pd, Rh, Ru, V, Sn, Sb, Bi, 2.5 ml of 100ppm Ni, 5 ml of 100ppm Cu, 1.25 ml of 10ppm Cd, 750µl of 10ppm Hg, 2.5ml of 1ppm Pb, 750µl of 1ppm As in to a 50 ml volumetric flask and make up to volume with diluent.

### 2.3.7 Preparation of Calibration Blank and Calibration Standards:
**Preparation of Calibration Blank:**
Transfer 0.5 ml of n-butanol and 2 ml of 100 ppb Indium Standard in to 10 mL volumetric flask and dilute up to the mark with diluent.

**2.3.8 Preparation of 0.5 J Calibration Standard:**
Transfer 0.5ml of 10J Standard in to 10 ml volumetric flask and add 2 mL of 10 ppm Indium Standard, 0.5 ml n-butanol and make up with diluent.

**2.3.9 Preparation of 1.0 J Calibration Standard:**
Transfer l.0ml of 10J Standard in to 10 ml volumetric flask and add 2 mL of 10 ppm Indium Standard, 0.5 ml n-butanol and made up with diluent.

**2.3.10 Preparation of 2.0 J Calibration Standard:**
Transfer 2.0 ml of 10J Standard in to 10 ml volumetric flask and add 2 mL of 10 ppm Indium Standard, 0.5 ml n-butanol and make up with diluent.

**2.3.11 Preparation of Test sample:**
Transfer 100 mg of sample for each preparation in to Teflon vessel (digestion tube) Add 3 mL of Conc HNO3, Load the vessels in microwave sample digester and start the digestion program as below

**Table 2-** Optimized Conditions

| Temperature[°C] | Pressure [bar] | Ramp (minutes) | Hold (minutes) | P[%] |
|---|---|---|---|---|
| 160 | 50 | 10 | 25 | 60 |
| 50 | 50 | 0 | 25 | 0 |
| 50 | 50 | 0 | 0 | 0 |
| 50 | 50 | 0 | 0 | 0 |
| 50 | 50 | 0 | 0 | 0 |

After completion of digestion carefully transfer the solution to 10 mL volumetric flask, rinse the teflon vessels with diluent and transfer the solution to the same volumetric flask. Add 2 mL of 100 ppb Indium Standard as internal standard, 0.5 ml n-butanol to each and make up to the mark with diluent.

**Preparation and Reagent Blank:**
Prepare as test sample procedure without addition of sample.

**Table 3- Calculation of J value:**
J value is calculated by the using the formula

**J (ppb) =** ____**PDE**___ **X 1000** (Dilution Factor=Dilution in ml/sample weight in gms)
          **Dilution factor**

| Element | PDE Value | Dilution factor | 1J value (wrt std in ppb) | 1J value (wrt sample in ppm) | 0.5J value (wrt std in ppb) | 0.5J value (wrt sample in ppm) | 2J value (wrt std in ppb) | 2J value (wrt sample in ppm) |
|---|---|---|---|---|---|---|---|---|
| Cadmium (Cd) | 2.5 | 100 | 25 | 2.5 | 12.5 | 1.25 | 50 | 5 |
| Molybdenum (Mo) | 10 | 100 | 100 | 10 | 50 | 5 | 200 | 20 |
| Rhodium (Rh) | 10 | 100 | 100 | 10 | 50 | 5 | 200 | 20 |
| Palladium (Pd) | 10 | 100 | 100 | 10 | 50 | 5 | 200 | 20 |
| Osmium (Os) | 10 | 100 | 100 | 10 | 50 | 5 | 200 | 20 |
| Platinum (Pt) | 10 | 100 | 100 | 10 | 50 | 5 | 200 | 20 |
| Iridium (Ir) | 10 | 100 | 100 | 10 | 50 | 5 | 200 | 20 |
| Ruthenium (Ru) | 10 | 100 | 100 | 10 | 50 | 5 | 200 | 20 |
| Copper (Cu) | 100 | 100 | 1000 | 100 | 500 | 50 | 2000 | 200 |
| Vanadium (V) | 10 | 100 | 100 | 10 | 50 | 5 | 200 | 20 |
| Lead (Pb) | 0.5 | 100 | 5 | 0.5 | 2.5 | 0.25 | 10 | 1 |
| Arsenic (As) | 0.15 | 100 | 1.5 | 0.15 | 0.75 | 0.075 | 3 | 0.3 |
| Mercury (Hg) | 1.5 | 100 | 15 | 1.5 | 7.5 | 0.75 | 30 | 3 |
| Tin(Sn) | 10 | 100 | 100 | 10 | 50 | 5 | 200 | 20 |
| Bismuth(Bi) | 10 | 100 | 100 | 10 | 50 | 5 | 200 | 20 |
| Antimony(Sb) | 10 | 100 | 100 | 10 | 50 | 5 | 200 | 20 |
| Nickel (Ni) | 50 | 100 | 500 | 50 | 250 | 25 | 1000 | 100 |

**Note:** Working solution 10 ppb is equivalent to 1 ppm with respect to sample.

**2.7 METHOD VALIDATION**
The validation of the elemental analysis method was carried out as per the international guidelines ISO/IEC 17025:2005**.** The parameters assessed were linearity, precision, accuracy, LOD and LOQ.

**2.7.1 Accuracy**

The accuracy of the method was evaluated in triplicate at three concentration levels 50%, 100% and 150 % of test concentration 10 mg/mL. The percentage of recoveries were calculated from the slope and Y-Intercept of the calibration curve. The accuracy study of metals was carried out in triplicate at 50%, 100%, & 150% of specification level (0.1%) to the Ingenol mebutate analyte concentration (1000 µg /mL).The percentages of recoveries for metals were calculated from the slope and Y- Intercept of the calibration curve

**2.7.2 Precision**

The precision of the elemental   method was evaluated by carrying out six independent preparations of Ingenol mebutate (each metal) test samples against internal standard and calculate the %RSD of metals.

**2.7.3 Linearity**

The purpose of the test for linearity is to demonstrate that the entire analytical system exhibits a linear response and is directly proportional over the relevant concentration range for the target concentration of the analyte. The linear regression data for the calibration plot is indicative of a good linear relationship between metal area and concentration over a wide range. The correlation coefficient was indicative of high significance.

**2.7.4 Limit of Detection & Limit of Quantitation**

The LOD can be defined as the smallest level of metal ion that gives a measurable response and LOQ was determined as the lowest amount of analyte that was reproducibly quantified. These two parameters were calculated using the formula based on the standard deviation of the response and the slope. LOD and LOQ were calculated by using equations, LOD=3.3×SD/S and LOQ=10×SD/s, where SD = standard deviation, S= slope of the calibration curve.

**2.7.5 Solution stability:**

The solution stability of Ingenol Mebutate in the heavy metals method of 10J Solution shall be used on the same day of preparation. (Valid up to 24 hrs)

**Table 4-** Limit of Detection and Limit of Quantification
LOD & LOQ Values

| Standard Concentration in ppb | Intensity | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Rh | Ir | Bi | V | Ru | Pd | Pt | Sb | Sn | Mo | Os |
| 10 | 234044.3 | 198158.2 | 254879.6 | 40914.8 | 37027.6 | 50979.1 | 75436.3 | 43499.8 | 35252.4 | 20025.6 | 56587.2 |
| 20 | 469357.1 | 394103.7 | 507877.4 | 78742.1 | 74564.1 | 102997.0 | 147383.4 | 85529.6 | 70286.8 | 40340.4 | 112479.5 |
| 25 | 569049.6 | 481786.5 | 612050.6 | 93861.3 | 90004.0 | 124394.7 | 179652.2 | 103438.8 | 85281.5 | 49027.9 | 136065.3 |
| 30 | 661969.4 | 561419.3 | 718631.4 | 109984.8 | 106153.0 | 146102.0 | 210510.8 | 122873.8 | 100048.8 | 57684.7 | 159386.5 |
| 40 | 886418.3 | 747979.8 | 949981.8 | 145118.0 | 140284.4 | 195102.7 | 280349.8 | 162924.1 | 132582.8 | 77083.9 | 211649.6 |
| 50 | 1084558.3 | 917704.9 | 1166623.4 | 177180.0 | 173266.8 | 240046.1 | 343275.0 | 200457.3 | 162956.1 | 94142.9 | 259725.7 |
| | | | | | | | | | | | |
| Std Error | 10672.899 | 7745.869 | 10419.225 | 1572.541 | 1401.043 | 2036.381 | 2450.578 | 1179.974 | 1338.071 | 847.216 | 2209.709 |
| Slope | 21149.526 | 17898.831 | 22632.526 | 3387.351 | 3379.315 | 4698.880 | 6679.525 | 3913.310 | 3173.192 | 1848.063 | 5048.871 |
| LOD | 1.514 | 1.298 | 1.381 | 1.393 | 1.244 | 1.300 | 1.101 | 0.905 | 1.265 | 1.375 | 1.313 |
| LOQ | 5.046 | 4.328 | 4.604 | 4.642 | 4.146 | 4.334 | 3.669 | 3.015 | 4.217 | 4.584 | 4.377 |

| Standard in ppb | Intensity | Standard in ppb | Intensity | Standard in ppb | Intensity | Standard in ppb | Intensity | Standard in ppb | Intensity | Standard in ppb | Intensity |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ni | | Cu | | As | | Cd | | Hg | | Pb |
| 50 | 312832.1 | 100 | 782324.6 | 0.150 | 348.0 | 2.50 | 6660.8 | 1.50 | 4182.6 | 0.50 | 7637.4 |
| 100 | 631212.1 | 200 | 1573525.8 | 0.300 | 697.7 | 5.00 | 13329.5 | 3.00 | 8257.7 | 1.00 | 14980.9 |
| 125 | 769934.6 | 250 | 1919644.7 | 0.375 | 785.0 | 6.25 | 16063.5 | 3.75 | 10107.6 | 1.25 | 18150.5 |

| 150 | 908459.4 | 300 | 2951868.6 | 0.450 | 929.4 | 7.50 | 19121.2 | 4.50 | 11971.3 | 1.50 | 21048.8 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **200** | 1211043.3 | 400 | 3921524.1 | 0.600 | 1250.7 | 10.00 | 25508.5 | 6.00 | 15723.6 | 2.00 | 27892.9 |
| **250** | 1490363.3 | 500 | 4844122.1 | 0.750 | 1551.4 | 12.50 | 31638.6 | 7.50 | 19492.6 | 2.50 | 34184.9 |
| **Std Error** | 10588.689 | Std Error | 212542.550 | Std Error | 26.493 | Std Error | 185.526 | Std Error | 107.731 | Std Error | 322.370 |
| **Slope** | 5864.973 | Slope | 10623.205 | Slope | 1979.345 | Slope | 2486.712 | Slope | 2536.716 | Slope | 13176.749 |
| **LOD** | 5.958 | LOD | 66.024 | LOD | 0.044 | LOD | 0.246 | LOD | 0.140 | LOD | 0.081 |
| **LOQ** | **18.054** | **LOQ** | **200.074** | **LOQ** | **0.134** | **LOQ** | **0.746** | **LOQ** | **0.425** | **LOQ** | **0.245** |

**Linearity:** Using calibration solutions calibration curves: $y = ax + b$, were determined, where $y$ is the signal intensity and $x$ is the know concentration of the given analyte in the calibration solution. The linearity of the calibration curve was considered acceptable when the correlation factor $R > 0.995$ (Table 5).

**Table 5-** Linearity Data

| Element | Correlation coefficient | Element | Correlation coefficient |
|---|---|---|---|
| Rh | 0.99509 | Cd | 0.99996 |
| Ir | 0.99547 | Pt | 0.99997 |
| Bi | 0.99586 | Hg | 0.99997 |
| Ni | 0.99777 | Sb | 0.99997 |
| Cu | 0.99978 | Sn | 0.99998 |
| V | 0.99996 | Pb | 0.99997 |
| As | 0.99985 | Mo | 0.99997 |
| Ru | 0.99995 | Os | 0.99992 |
| Pd | 0.99997 | | |

**Table 6-** Accuracy Data

| Element | Test + 50% spike | % Recovery | Test + 100% spike | % Recovery | Test +1 50% spike | % Recovery |
|---|---|---|---|---|---|---|
| Sample Weight | 101.77 mg | NA | | | | |
| Rh | 2.657 | **104** | 5.761 | **100** | 12.013 | **102** |
| Ir | 2.715 | **105** | 5.961 | **99** | 12.061 | **104** |
| Bi | 2.625 | **102** | 6.546 | **85** | 11.818 | **102** |
| Ni | 13.279 | **103** | 37.287 | **124** | 55.584 | **103** |
| Cu | 33.257 | **105** | 71.264 | **107** | 108.643 | **106** |
| V | 4.887 | **99** | 9.941 | **102** | 14.963 | **101** |
| As | 0.059 | **100** | 0.115 | **110** | 0.178 | **103** |
| Ru | 4.838 | **99** | 9.714 | **100** | 14.644 | **100** |
| Pd | 4.636 | **101** | 9.153 | **102** | 13.751 | **102** |
| Cd | 0.998 | **101** | 1.982 | **107** | 2.986 | **105** |
| Pt | 4.449 | **101** | 9.056 | **100** | 13.296 | **103** |
| Hg | 0.551 | **100** | 1.108 | **106** | 1.664 | **106** |
| Sb | 4.033 | **101** | 8.064 | **109** | 12.270 | **105** |
| Sn | 5.31 | **101** | 10.168 | **103** | 14.929 | **103** |
| Pb | 0.262 | **89** | 0.539 | **95** | 0.755 | **92** |
| Mo | 5.132 | **101** | 10.309 | **103** | 15.417 | **102** |
| Os | 4.789 | **100** | 9.714 | **98** | 14.409 | **102** |

## III.    Results and Discussion

To establish and validate an efficient method for elemental analysis of Ingenol mebutate in Active pharmaceutical ingredients, preliminary tests were performed. Different spectrometric conditions were employed for the analysis of the Ingenol mebutate active pharmaceutical ingredients. Finally the analysis was performed by using diluent**:** Conc HNO3 and   Conc HCl in the ratio of 31mL and 17Ml into 1000 mL of Milli Q water The proposed method was optimized to give very reliable results .The optimized spectrometric operating  conditions were given in    table 1.

Precision was evaluated by a known concentration of metal ions of Ingenol mebutate was injected six times and corresponding areas were recorded and % RSD was calculated and found within the limits. The low % RSD value was indicated that the method was precise and reproducible. Accuracy of the method was proved by performing recovery studies of Ingenol mebutate for each metal ions thrice at 50%, 100% and 150%, level. Recoveries of each metal  namely (Cd, Pb, As ,Hg ,Ir, Pd, Pt, Rh, Ru ,Os, Mo, Ni, V, Cu, Sn, Sb, Bi  ranges from 75% to 126% in proposed method and the results were shown in the (Table 6).

Linearity was established by analyzing different concentrations for metals of ,Ir, Pd, Pt, Rh, Ru ,Os, Mo, V,, Sn, Sb, Bi 10%, 20%, 25%, 30%, 40% and 50% level and Ni, Cu, As ,Cd, Hg & Pb 50%,100%,125% 150%,200%,250% of Ingenol mebutate metal ions. The calibration curve was plotted with the area obtained versus concentration of Ingenol mebutate metal ions, In the present study concentrations were chosen ranging between 10ppb to 250 ppb of Ingenol mebutate metal ions. The linear regression data for the calibration plot is indicative of a good linear relationship between peak area and concentration over a wide range. The correlation coefficient was indicative of high significance and the results were shown in the (Table 5)

## IV. Conclusion

A new ICP-MS method has been developed for seventeen trace metals of Ingenol mebutate namely Cd, Pb, As ,Hg ,Ir, Pd, Pt, Rh, Ru ,Os, Mo, Ni, V, Cu, Sn, Sb, Bi in Active pharmaceutical in gradients. The developed method was validated and it was found to be selective, precise, accurate and linear it can be used for the routine analysis of Ingenol mebutate in Active pharmaceutical ingredients. Several parameters have been taken into account and evaluated for the validation of method: the limit of detection ranged between (0.0044–66.024) for the 17 metals studied ensures the minimum limit of quantification required for 17 Determination of metals in Ingenol mebutate by ICP-MS (0.245–0200.074); good linearity (correlation factor $R > 0.995$).

The validation studies were carried out in accordance with international guidelines ISO/IEC 17025:2005. Finally it was concluded that the method is simple, selective, and cost effective and has the ability to detect all seventeen metals of Ingenol mebutate found in Active pharmaceutical in gradients.

Quantitative analysis by ICP-MS has been proven to be a powerful tool for rapid determination of elements and the method is particularly useful for the analysis of elemental impurity samples.

## Acknowledgements

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