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# Quantitation of Protopine in *Fumaria parviflora* L. Whole Plant Powder by High-Performance Liquid Chromatography

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Present method describes a high performance liquid chromatographic determination of protopine from *Fumaria parviflora* Linn. The separation was carried out on Cosmosil ($C_{18}$) (250 mm × 4.6 mm, i.d.) 5 μ column. The mobile phase was buffer: methanol in the volume ratio of (50:50) (v/v). The detection and quantification was carried out using a UV-visible detector at 291 nm. Linear concentration range of protopine was observed to be 0.0533- 0.2133 μg $mL^{-1}$. The developed method was validated to determine its accuracy, precision and stability by carrying out linearity and stability experiments. The method developed is simple, fast, accurate and precise and hence can applied for routine quality control analysis of *Fumaria parviflora* Linn. whole plant powder and quantitative determination of protopine.

**Key Words: HPLC, Protopine, *Fumaria parviflora* L., UV detector.**

## INTRODUCTION

Natural products isolated from the leaves, stems, roots and seeds of plant *Fumaria parviflora* L, of Fumariaceae family, are mainly alkaloids. Of these, protopine is a predominant compound in the whole plant. Protopine, a known purified component from several botanical drugs has been examined for its antithromobotic, antiinflammatory, antiociceptive also inhibits K"A"T"P channel currents in human embryonic kidney cells (HEK-293)[1,2]. From the whole plant of *Fumaria parviflora*, coptisine, corytuberine, cryptopine, fumaricine, fumariline, fumaritine, fumarophycine, O-methylfumarophycine, palmatine, parfumine, protopine, sinactine, stylopine and N-methylstylopine, citric, coumaric, ferulic, fumaric, malic, 3-hydroxybenzoic, protocatechuic acid and caffeic acid[3].

The literature reveals that there is no high-performance liquid chromatographic method is available for determination of protopine in *Fumaria parviflora* whole plant powder. A simple, rapid, economical, precise and accurate HPLC method has been established for determination of protopine in *Fumaria parviflora* whole plant powder. This method can be used for phytochemical profiling of *Fumaria parviflora* whole plant powder and quantitation of protopine. This enables identification of whole plant material by matching the phytochemical profile of the market product with a standard laboratory sample under the same experimental conditions. It also

enables the quantitation of protopine, thereby enabling the confirmation of the label claim of protopine containing products.

## EXPERIMENTAL

The plant Fumaria parviflora was collected from Lucknow, Punjab and Western Ghats of Maharashtra and was authenticated by National Botanical Research Institute, India. Standard protopine was procured from reputed research centre.

Thermo Separation Products, P100 HPLC isocratic pump fitted with 20 µL loop and Thermo Separation Products, UV 100 variable wavelength detector were used. The recorder used was Indtech, Oracle2 chromatography software.

Triethyl amine, methanol, orthophosphoric acid used was of spectroscopic grade.

### Preparation of drug solutions

**Standard drug solution:** Accurately weighed 100 mg pure standard of protopine (99.99 %) was transferred to 100 mL volumetric flask. The drug was dissolved in methanol and diluted upto the mark and mixed well. This gave a standard stock solution of strength 1000 µg $mL^{-1}$, by making suitable dilution; 10 µg $mL^{-1}$ solution was also prepared. All dilutions were performed in standard volumetric flasks.

**Preparation of buffer:** 6 mL of triethyl amine was added to 1000 mL of water and pH was adjusted to 7.0 with 50 % orthophosphoric acid in.

### Method validation[4-6]

**Chromatographic condition:** The separation was carried on Cosmosil ($C_{18}$) (250 mm × 4.6 mm, i.d.) 5 µ column. The mobile phase was buffer:methanol in the volume ratio of (50:50) (v/v). The flow rate of the mobile phase was maintained at 1.0 mL/min and the detector wavelength was set at 291 nm. The rise time of the detector was set at 3.0 s. The AUFS (area under full scale) of the detector was set at 0.02. The attenuation was set at 5 and the chart speed at 1.0 cm/min.

**System suitability test:** The system suitability test was performed by injecting a standard protopine 5 times. Mean, standard deviation and coefficient of variance were calculated.

**Linearity of detector response:** Solutions containing protopine at 7 different concentrations were prepared in methanol. Each of these solutions (20 µL) was injected into the system and the detector response for the different concentrations was measured[7]. The area of the peak from the drug was measured from each concentration and a graph was plotted of peak area against amount of protopine. The plot was linear in the range 0.0533 to 0.2132 µg. The experiment was performed three times and the mean was used for the calculations. The data was analyzed by linear regression least-squares fitting[8]. The statistical data obtained are given in Table-1.

### Precision studies

**Spotting repeatability/instrument precision:** This is carried out by injecting standard protopine solution (0.133 µg $mL^{-1}$) on to the chromatographic system.

TABLE-1
LINEARITY DATA

| | |
|---|---|
| Intercept | 19486.57 |
| Standard error of *y* estimate | 12903.09 |
| $R^2$ (Regression coefficient) | 0.999952 |
| Number of observations | 7 |
| Degrees of freedom | 5 |
| *x* coefficient (slope) | 29584883 |
| Standard error of coefficient | 91464.93 |

Twenty microlitre of this solution was injected into the system 7 times in the same chromatographic conditions; the detector response for this peak was monitored using a UV detector set at a wavelength of 291 nm. The chromatograms were recorded and the peak areas were recorded. The mean, standard deviation and coefficient of variation (%) of all these parameters were calculated. The results of spotting repeatability/ instrument precision are given in Table-2.

TABLE-2
RESULTS OF INJECTION REPEATABILITY/INSTRUMENT PRECISION

| Conc. of drug (μg/mL) | Peak area | Response factor |
|---|---|---|
| 0.1333 | 3954784 | 29668295 |
| 0.1333 | 3964812 | 29743526 |
| 0.1333 | 3956895 | 29684134 |
| 0.1333 | 3963256 | 29731853 |
| 0.1333 | 3954732 | 29667907 |
| 0.1333 | 3961598 | 29719415 |
| 0.1333 | 3964895 | 29744149 |
| Mean | 3960139 | |
| SD | 4559.582 | |
| COV | 0.115137 | |

**Intra-assay/within day precision:** This experiment is carried out in laboratory, on one day using three different concentration levels with three repetitions each. Three sets of experiment were carried out, each set containing three levels of 0.10, 0.133 and 0.160 μg of protopine. Solution for each of the levels were independently prepared, starting from separate weighing to obtain these concentrations of 0.10, 0.133 and 0.160 μg after injecting 20 μL of each of these solutions. The detector response for these peaks was monitored using a UV detector set at a wavelength of 291 nm. This experiment was carried out thrice and the mean, standard deviation, coefficient of variation and response factors for each level were calculated. The results are tabulated in Table-3.

**Intermediate precision:** Intermediate precision is the precision obtained when multiple analysts or using multiple instruments or on multiple days in laboratory the assay was performed. Different lots of reagents and multiple lots of plates may also be used to prove intermediate precision. The results obtained by this experiment

TABLE-3
RESULTS OF INTRA-ASSAY/WITHIN DAY PRECISION

| Day 1 | Conc. of drug taken (µg/cm³) | | | Peak area | | | Mean | SD | COV % |
|---|---|---|---|---|---|---|---|---|---|
| Obs. No. | 1 | 2 | 3 | 1 | 2 | 3 | | | |
| 1 | 0.107 | 0.999 | 0.101 | 3171844 | 3179895 | 3191822 | 3181187.0 | 10051.47 | 0.315966 |
| 2 | 0.133 | 0.130 | 0.129 | 3974784 | 3954722 | 3964812 | 3964772.6 | 10030.93 | 0.253001 |
| 3 | 0.160 | 0.160 | 0.159 | 4757780 | 4857845 | 4786812 | 4800812.3 | 51480.66 | 1.072332 |

are used to identify which of the above factors contribute significant variability to the final result. The experiment carried out for proving intermediate precision was analysis on different day. The mean, standard deviation and coefficient of variation were calculated. The peak area ratios were comparable to those of the intra-assay results. The results are given in Table-4.

TABLE-4
RESULTS OF INTERMEDIATE PRECISION ON DIFFERENT DAY

| Day 2 | Samples prepared on a different day | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Conc. of drug taken (µg/cm³) | | | Peak area | | | Mean | SD | COV % |
| Obs. No. | 1 | 2 | 3 | 1 | 2 | 3 | | | |
| 1 | 0.999 | 0.101 | 0.103 | 3161844 | 3201858 | 3141865 | 3168522.3 | 30548.98 | 0.964140 |
| 2 | 0.128 | 0.132 | 0.133 | 3958684 | 3914745 | 3994787 | 3956071.9 | 40084.87 | 1.013249 |
| 3 | 0.160 | 0.160 | 0.159 | 4813635 | 4698699 | 4757725 | 4756686.3 | 57475.04 | 1.208300 |

Accuracy of method can be proved by recovery experiment. Standard addition method was used for performing recovery experiment. Known amounts of protopine standard were added to pre-analyzed whole plant powder at three different concentration levels, *i.e.* 120, 140 and 160 % of the amount of drug present in the whole plant powder (the external standard addition technique). The method was repeated seven times starting from weighing of whole plant powder. After finding the mean, standard deviation, coefficient of variation % recovery for each level was calculated. Data obtained from recovery experiment is tabulated in Table-5.

TABLE-5
RESULTS FROM RECOVERY EXPERIMENT

| Level | Amount of protopine acid in whole plant powder (mg) | Amount of protopine acid std added to whole plant powder (mg) | Total amount of protopine found (mg) | Recovery (%) | SD | CV (%) |
|---|---|---|---|---|---|---|
| 0 | 13.52 | 0.0 | 13.52 | 101.60 | 34153.43 | 0.849896 |
| 1 | 12.98 | 1.0 | 16.50 | 101.90 | 42551.09 | 0.871115 |
| 2 | 14.50 | 2.0 | 19.20 | 101.30 | 111021.30 | 1.940260 |
| 3 | 14.27 | 3.0 | 21.30 | 100.00 | 86356.08 | 1.351295 |

**Use of the method for phytochemical fingerprinting of *Fumaria parviflora* whole plant powder and for determination of protopine in whole plant powder**

**Sample preparation:** The whole plant of *Fumaria parviflora* was dried at room temperature and then ground in a mixer to a fine powder, which passed through an ASTM BSS 85 mesh size. Powder (10 g) was accurately weighed, placed in a 100 mL volumetric flask and methanol (100 mL) was added to the flask. After shaking, the flask was left overnight at room temperature (28 ± 2 ºC). The contents of the flask was then filtered through a Whatman No. 41 paper and the clear supernatant was collected in another clean, dry, stoppered conical flask (solution A; 10,000 µg $mL^{-1}$). This was then serially diluted with methanol to give a 10 µg $mL^{-1}$ solution. This solution (solution B) was used for the assay experiment.

**Assay procedure:** The sample working solution (20 µL) was injected into the system under the standard conditions and the area of protopine peak was measured. The amount of protopine present in this solution was then determined using peak area values from the standard and the sample solution. The assay procedure described earlier was repeated seven times starting from weighing of the whole plant powder.

The amount of powder weighed, the area of the protopine peak and the percentage content of protopine in whole plant powder are given in Table-6.

TABLE-6
RESULTS FROM ASSAY EXPERIMENT

| Sample No. | Amount of powder (mg) | Volume (mL) | Peak area | | | Found (mg) | Amount present (%) |
|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | Average | | |
| 1 | 10002.3 | 100.0 | 4036528 | 4039365 | 4037946.5 | 13.52 | 0.1352 |
| 2 | 10003.5 | 100.0 | 4051456 | 4025685 | 4038570.5 | 13.52 | 0.1352 |
| 3 | 10008.6 | 100.0 | 3945625 | 4012586 | 3979105.5 | 13.32 | 0.1332 |
| 4 | 10002.5 | 100.0 | 3859125 | 3892515 | 3875820.0 | 12.98 | 0.1298 |
| 5 | 10009.1 | 100.0 | 4158628 | 4210358 | 4184493.0 | 14.00 | 0.1400 |
| 6 | 10001.1 | 100.0 | 4251691 | 4268912 | 4260301.5 | 14.27 | 0.1457 |
| 7 | 10008.9 | 100.0 | 4312586 | 4356123 | 4334354.5 | 14.50 | 0.1450 |
| | | | | | Mean | 13.73 | 0.14 |
| | | | | | SD | 0.55 | 0.01 |
| | | | | | % RSD | 3.97 | 3.97 |

## RESULTS AND DISCUSSION

The proposed method is based on the separation of the active constituent *i.e.* protopine from the raw material by high performance liquid chromatography method using a C-18 column. The measurement was done at 291 nm. The drug concentration was linear in the range of 0.0533 to 0.2133 µg $mL^{-1}$. The lowest level of quantification was observed to be 0.0133 µg $mL^{-1}$ which indicates the sensitivity of the method.

The coefficient of variation for system suitability experiment was observed to be less than 2 % for retention time, peak asymmetry, tailing factor and peak area ratio indicating suitability of the system. Instrument precision, intra assay precision and intermediate precision were measured to evaluate the precision of the method.

Determination of intermediate precision entailed analysis on a different day and by a different analyst. The values of standard deviation and COV were calculated. COV was in the range 0.960 to 1.20 % for intraday precision and 0.31 to 1.07 % for inter-day precision. These low values of coefficient of variation are indicative of high precision of the method.

The accuracy of the method was established by means of a recovery experiment. Known amounts of oleanolic acid were added to pre-analyzed whole plant powder at three different concentration levels, *i.e.* 120, 140 and 160 % of the amount of drug in the whole plant powder (the external standard addition technique). The fortified samples were then analyzed seven times. The results of recovery analysis are given in Table-5. The mean recovery was close to 101.74 %, which indicates the accuracy of the method. The low values of COV for seven replicate analyses are indicative of precision of the method.

The robustness of the method was studied, during method development, by determining the effects of small variation, of mobile phase composition (± 5 %), the ratio of methanol was initially changed from 50 to 45 mL and then from 50 to 55 mL there was slight variation in the retention time of protopine but the peak area in both the cases was more or less the same indicating the robustness of the method.

Regional variation was carried out to find out which of the regions contained high amount of protopine. It was clearly seen that the plant material collected from Lucknow contained maximum amount of protopine indicating that the plant material *Fumaria parviflora* when collected from Lucknow would be more efficacious than from the other regions.

## Conclusion

The proposed method is simple, rapid, selective, sensitive and inexpensive and can be used for routine quality-control analysis of *Fumaria parviflora* whole plant powder and for quantitative determination of protopine in whole plant powder.

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