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Reversed-phase high-performance liquid chromatography method for impurity profiling of generic drug Calcium Orotate

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New reversed-phase high-performance liquid chromatography method has been developed and validated for the identification of impurity in calcium orotatedihydrate (CaOD) drug. The developed method has been achieved superior resolution between the impurity and CaOD using Alltima C-18 column (250 X 4.6 mm, 5 µm). The method has been established by isocratic mobile phase comprising of 0.01 mol/L of potassium dihydrogen orthophosphate, pH adjusted to 2.7 with orthophosphoric acid and acetonitrile in a ratio of 98:2 v/v. The eluted impurity at the retention time of 6.02 min has so far not been reported in earlier methods of analysis. According to International council for harmonization guidelines, the method has been validated with respect to accuracy, precision, specificity, linearity, range, robustness, system suitability, limit of detection and limit of quantification. The percentage recovery of CaOD and uracil is found in the range between 98% and 102%. The linearity data has shown that the method is found to be linear for CaOD and uracil from 25% to 150% level and the correlation coefficient is found to be more than 0.999. The reported method is not affected by small but deliberate changes in the parameters of the method.

Keywords: Calcium orotatedihydrate, Liquid chromatography, Mobile-phase, Method validation

In general, human body demands calcium supplement that helps to build strong bones and prevents osteoporosis[1-3]. In order to maintain the normal functioning of cells, nerves, bones, muscles etc. one should have the calcium content as per the recommended dietary allowance according to their age. Suppose there is a lack of calcium in the blood, then the body will consume calcium from bones, and thus reduces the strength of bones[4-5]. The calcium content in our body is helped to prevent weight gain and sustaining healthy weight[6-8]. The effects of reduced calcium levels indicates emergency starvation mode and starts to leach calcium into the bloodstream. Orotic acid known as pyrimidine carboxylic acid supports the transport of calcium through cellular membrane structures which facilitates the intracellular uptake of calcium, particularly in bone[9]. Calcium orotate is a source of superior calcium supplement and absolutely free from side effects. It is an essential raw material for making the genetic substances deoxyribonucleic acid and ribonucleic acid.

Calcium orotatedihydrate (CaOD) is also a calcium supplement which helps in the maintenance of healthy cartilage. CaOD is chemically termed as calcium 1,2,3,6-tetrahydro-2,6-dioxopyrimidine-4-carboxylate and it is a calcium salt of orotic acid. The appearance of CaOD is a white to almost white crystalline powder with very slightly soluble in water and practically insoluble in alcohols. The usage of CaOD drug includes i) hip joint plastic surgery, ii) hypoparathyroidism, iii) premenstrual syndrome, iv) controlling weight, v) prevent low blood calcium level, etc. Since CaOD has high affinity to penetrate complex cellular membrane, it is generally suggested as calcium supplement for people who find it problematic to get sufficient calcium, nursing mothers and pregnant women[10-12]. This medication is also used to treat or prevent low blood calcium levels in people who do not get enough calcium from their diets. The usage of CaOD drug to regain healthy bone from osteoporosis is represented in Fig. 1. The drug CaOD is manufactured by two step processes in which the first step, uracil is converted into orotic acid and the second step yields CaOD by treating the orotic acid with calcium chloride.

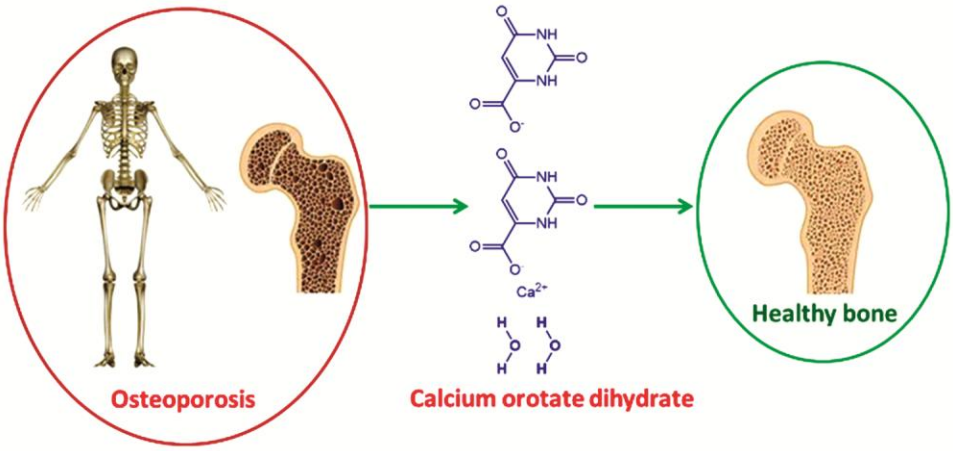

Fig. 1 — Applications of CaOD drug in osteoporosis

Investigation of impurity profile in a drug molecule is essential for the manufactured products in pharmaceutical industry,[13-16] High-performance liquid chromatography (HPLC) is a widely used instrument to analyse the compound purity and profile comprising of both known and unknown impurities[17-22]. The literature survey reveals that there is no method available for the quantification of impurity exists in CaOD bulk drug. Since, the drug is not included in any official pharmacopeia, an attempt was taken to develop a simple cost effective method for quantification of impurity in CaOD drug and also the method was validated according to the International council for harmonization (ICH) guidelines. The developed RP-HPLC analytical method is rapid, accurate, precise, and reproducible. Hence, the method can be applied for routine quality control analysis for the estimation of impurity in CaOD bulk drug.

Materials and Methods

Reagents and Instrumentation

Potassium dihydrogen orthophosphate (KH_2PO_4) and orthophosphoric acid were purchased from Merck India Ltd. Uracil was purchased from Sigma Aldrich Co. Calcium orotatedihydrate (CaOD) was procured from the market in India which was manufactured by renowned manufacturing company. HPLC grade acetonitrile and water were received from RANKEM.

Agilent HPLC system (Model: 1220) equipped with a UV detector and binary pump is used to investigate the related substance analysis of CaOD drug. The instrument has an auto injection facility and OpenLab CDS software was used to integrate the chromatograms. In order to determine the best conditions for resolving all the components present in CaOD drug, Alltima C-18 column (250 X 4.6 mm, 5µm) was used for the HPLC analysis. The column temperature was maintained at 25 °C for the whole duration of analysis.

Preparation of mobile phase

The buffer solution was prepared by the dissolution of 0.01 M (1.36 g) KH_2PO_4 in 980 mL of HPLC grade water and the pH was adjusted to 2.7 using orthophosphoric acid followed by a final dilution to a volume of 1000 mL with HPLC grade water. The mobile phase was prepared by mixing together the buffer solution and HPLC grade acetonitrile at a ratio of 98:2 v/v. The prepared mobile phase was filtered through a 0.45 µ filter paper and degassed prior to use.

Standard and sample preparation

Stock solution of standard CaOD was prepared by the dissolution 50.0 mg of substance in 25 mL of hot water, followed by cooled and made up to 50 mL with HPLC grade water. Further dilution was carried out by pipetting out of 5 mL of the stock solution into 50 mL standard flask and made up to the mark using water (Stock solution A). Similarly, stock solution of uracil was prepared by dissolving 50.0 mg of substance in 25 mL of water and made up to 50 mL with water. A volume of 5 mL of the said solution of uracil was diluted to 50 mL with water (Stock solution B). Reference solution was prepared by pipetting out 1.5 mL of stock solution A and 1.0 mL of stock solution B in to a 100 mL volumetric flask and made up to the

mark with mobile phase. CaOD test solution was prepared by dissolving about 50.0 mg of substance in 25 mL of hot water, allowed to cool steadily and then made up to 100 mL with mobile phase.

Spiked sample preparation

Spiked solution was prepared by dissolving about 50 mg of CaOD substance in 25 ml of water taken in a 100 mL standard flask and dissolved the content by heating. To this solution, 0.5 mL of stock solution B was added followed by diluted up to 100 mL with mobile phase.

Chromatographic condition

An isocratic system of mobile phase consists of buffer and acetonitrile at a ratio of 98:2 v/v was found to be optimized condition for the related substance analysis of CaOD drug. The injection volume was fixed to 10 µL for both the standards and samples. The flow rate of mobile phase was set to 1.0 mL/min through C-18 column and UV detection was monitored at a wavelength of 205 nm. The HPLC column was conditioned with mobile phase for 1 h prior to analysis.

Results and Discussion

HPLC method development

Identification of impurity is the process of elucidating the nature of known or unknown materials exist in a drug sample. It is important from a pharmaceutical point of view to quantify both CaOD and the impurities present in the final product. Developing a method to investigate the impurities will aid in the quality control process of the drug molecule. CaOD is slightly soluble in water and practically insoluble in methanol and ethanol. The pKa value of orotic acid is 2.83[Ref. 23] which suggest a pH of buffer solution should be 2.83±1. In order to find out the optimum mobile phase condition to get neat chromatograms, the method was screened for various compositions of buffer and acetonitrile.

Three different combination of mobile phases were prepared by mixing buffer and acetonitrile in a ratio of i) 90:10 v/v, ii) 95:5 v/v and iii) 98:2 v/v. Finally, 0.01 M KH_2PO_4, pH adjusted to 2.7 with orthophosphoric acid and acetonitrile in a ratio of 98:2 v/v showed sharp chromatographic peaks with superior resolution between CaOD and impurity. Similarly, trials were carried out to find out the maximum absorbance (λ_{max}) of CaOD drug and impurity at different wavelengths such as i) 275 nm, ii) 258 nm, iii) 210 nm and iv) 205 nm. UV spectra of standard calcium orotatedihydrate and uracil were shown in Supplementary Data, Fig. S1 and Fig. S2, respectively. Fig. S1 displays two maximum absorbance at 206.82 nm and 277.28 nm, whereas Fig. S2 shows the absorbance maximum at 201.45 nm and 258.45 nm.

The analytical results demonstrated that adequate response obtained for both CaOD drug and impurity at λ_{max} of 205 nm with selected mobile phase condition of 98:2 v/v. Typical HPLC chromatograms of standard and sample calcium orotatedihydrate are presented in Fig. 2 and Fig. 3, respectively. A sharp peak obtained at the retention time of 4.83 min was corresponded to the CaOD. Along with the main peak, one additional peak was eluted at the retention time of 6.02 min.

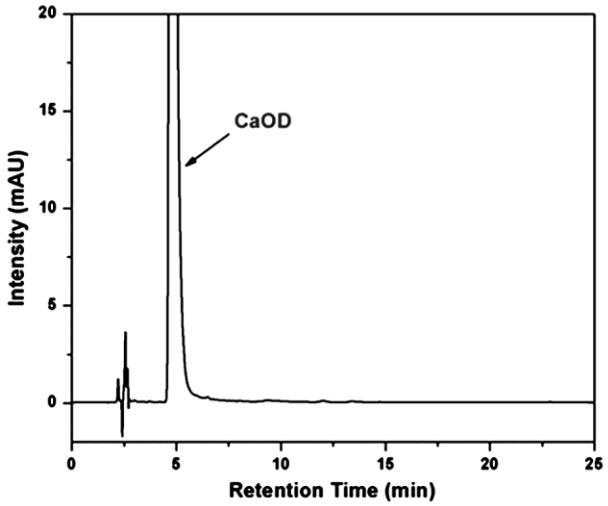

Fig. 2 — Chromatogram of standard CaOD

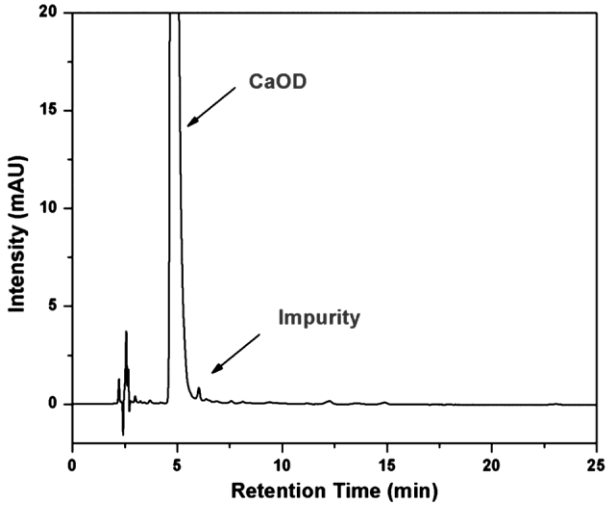

Fig. 3 — Chromatogram of sample CaOD

Based on the synthetic route of CaOD, it may be assumed that the eluted impurity in Fig. 3 is either uracil or unknown impurity. Hence, uracil was analysed by the same method and the retention time was found to be 6.02 min. Further, uracil was spiked in to CaOD sample and analysed under the same condition of chromatographic system. Fig. 4 depicts the HPLC chromatogram of test solution spiked with 0.1 % uracil. From the results, it may be concluded that the eluted impurity is uracil. The HPLC chromatogram of reference solution C is presented in Fig. 5. It can be seen from Fig. 5 that a good resolution was observed between CaOD and uracil. Further, the developed method has been validated according to ICH guidelines. Two drug samples were tested for impurity profiling and both are found to be similar impurity profile.

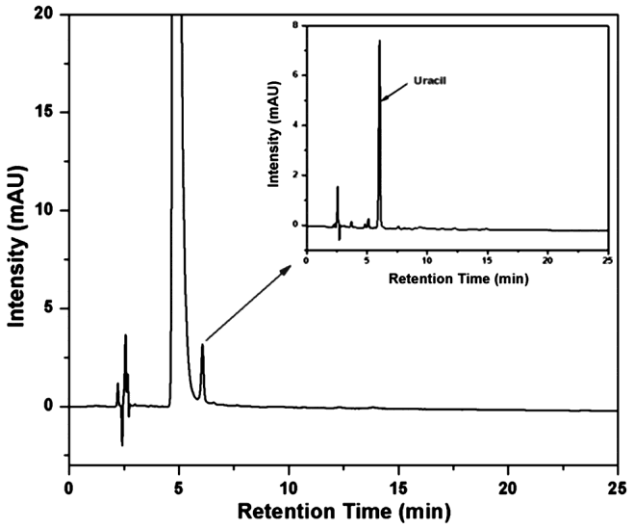

Fig. 4 — Chromatogram of test solution spiked with uracil

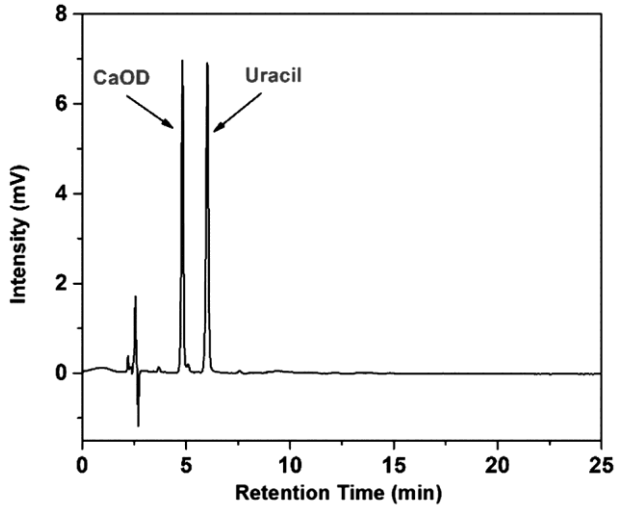

Fig. 5 — Chromatogram of reference solution C

Validation studies

Specificity

In order to evaluate the absence of interference in CaOD drug, the specificity of HPLC method was conducted. To prove the specificity of the developed analytical method, blank (mobile phase), test solution and reference solution C were injected into the chromatographic system. There were no interference peaks observed at retention time of CaOD and uracil. From this experiment it can be concluded that the method was specific with respect to any interference peak.

Accuracy

Accuracy of the method was demonstrated by injecting the standard at 50%, 100% and 150% level with respect to the analyte concentration. Accuracy solution was prepared in triplicate at each level and injected each preparation once into chromatographic system. The accuracy data received from the chromatograms of CaOD and uracil are presented in Table 1 and Table 2 respectively. The percentage recovery of CaOD and uracil was found in the range between 98 % and 102 %. Hence, the method is found to be accurate.

Linearity and range

The linearity of an analytical procedure is directly proportional to the analyte concentration of a sample[24]. From the stock solution of CaOD, five different concentrations (0.37 µg/mL, 0.75 µg/mL, 1.13 µg/mL, 1.50 µg/mL and 2.25 µg/mL) were prepared. All the solutions were filtered through 0.45 µ filter paper before loaded in to the chromatographic system. To evaluate the linearity, the resultant average peak areas of CaOD and uracil were plotted against the respective concentrations. The linearity data of the CaOD drug and uracil were provided in Supplementary Data, Table S1 and Table S2, respectively. The correlation coefficient was found to be more than 0.999. The calibration data of CaOD and uracil are displayed in Fig. 6. The results indicate that the method was found to be linear for the drug CaOD and uracil from 25% level to 150% level with respect to the analyte concentration.

Table 1 — Recovery results of CaOD

Spike level (%)	Sample concentration (μg/mL)	Amount of CaOD added (μg/mL)	Total theoretical Amount of CaOD (μg/mL)	Amount of CaOD recovered (μg/mL)	Recovery (%)	Statistical parameters
	0.5024	0.7536	1.256	1.2668	100.86	Mean: 100.69
50	0.5024	0.7536	1.256	1.2697	101.09	SD: 0.51
	0.5024	0.7536	1.256	1.2575	100.12	RSD: 0.50
	0.5024	1.5072	2.0096	2.0051	99.78	Mean: 99.65
100	0.5024	1.5072	2.0096	2.0060	99.82	SD: 0.26
	0.5024	1.5072	2.0096	1.9966	99.35	RSD: 0.26
	0.5024	2.2608	2.7632	2.7420	99.23	Mean: 99.48
150	0.5024	2.2608	2.7632	2.7562	99.75	SD: 0.26
	0.5024	2.2608	2.7632	2.7481	99.45	RSD: 0.26

SD - Standard deviation; RSD - Relative standard deviation

Table 2 — Recovery results of uracil

Spike level (%)	Sample concentration (mg/mL)	Amount of uracil added (μg/mL)	Amount of uracil recovered (μg/mL)	Recovery (%)	Statistical parameters
	0.5016	0.5084	0.5033	99.01	Mean: 99.72
50	0.5016	0.5084	0.5026	98.86	SD: 1.36
	0.5016	0.5084	0.5149	101.28	RSD: 1.36
	0.5016	1.0168	1.0118	99.51	Mean: 100.19
100	0.5016	1.0168	1.0162	99.94	SD: 0.83
	0.5016	1.0168	1.0281	101.11	RSD: 0.83
	0.5016	1.5252	1.5356	100.68	Mean: 100.23
150	0.5016	1.5252	1.5292	100.26	SD: 0.46
	0.5016	1.5252	1.5215	99.76	RSD: 0.46

SD - Standard deviation; RSD - Relative standard deviation

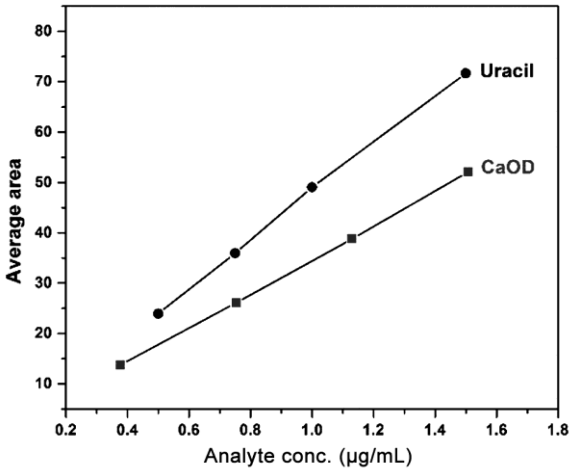

Fig. 6 — Linearity calibration of CaOD and uracil

Precision

The precision repeatability of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the same operating conditions over a short interval of time. The precision was tested with the same sample by taking six weights and injected each one. Table 3 presents the precision data of CaOD and uracil, where the %RSD was found to be less than 2.0 for both CaOD and uracil.

Table 3 — Precision repeatability data of CaOD and uracil

Test solution	Area of CaOD	Area of uracil
Sample 1	17521.490	5.214
Sample 2	17568.129	5.353
Sample 3	17541.049	5.384
Sample 4	17535.102	5.419
Sample 5	17458.279	5.371
Sample 6	17522.998	5.158
Average	17524.508	5.321
% RSD	0.2	1.9

RSD - Relative standard deviation

Limit of detection (LOD) and limit of quantification (LOQ)

The determination of signal-to-noise ratio is performed by comparing the measured signals of the analytes in the reference solution with those of blank runs in mobile phase and establishing the minimum concentration at which the analyte can

Table 4 — LOD and LOQ results

Parameters	CaOD	Uracil
LOD (%)	0.008	0.005
LOQ - Theoretical (%)	0.028	0.018
LOQ - Practical (%)	0.028	0.018

be reliably detected. The LOD was calculated with the signal-to-noise ratio 3:1. The determination of signal-to-noise ratio is performed by comparing the measured signals of the analytes in the reference solution with those of blank runs in mobile phase and establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision. Table 4 shows the LOD and LOQ values of both CaOD and uracil. The LOQ was calculated with the signal-to-noise ratio 10:1.

Solution stability

Solution stability was established by leaving the blank, reference and test solution in tightly closed volumetric flask at room temperature on a laboratory bench for 48 h. The content of CaOD and uracil was checked after 24 h and 48 h against the freshly prepared. Day to day area changes were observed with respect to initial area (0^{th} h) and % RSD was found to be less than 1.0. This Low value of RSD indicates that the reference and test solutions are stable up to 48 h.

System suitability

System performance of the developed HPLC method was investigated by injecting standard solutions. In order to assess the suitability of the system, parameters such as theoretical plates (N) and tailing factor (Tf) were determined. The results of such system suitability parameters for CaOD and uracil are presented in Table 5. It can be concluded from the data that the parameters indicate good performance of the system for the related substance analysis of CaOD drug.

Robustness

Robustness of the method was performed by deliberate modification of flow rate and variation of pH in a mobile phase composition separately by keeping other parameter intact. Two types of variations such as (i) change in flow rate by ± 0.2 mL /min and (ii) change of pH in mobile phase composition by ±0.3 were carried out. The effects of the change in parameters were studied and found that the method was well adapted to accept slight changes in the retention times as shown in Table 6. The analytical results proved that the method is robust.

Table 5 — System suitability studies of CaOD and uracil

Parameters	Results of CaOD		Results of uracil		Allowable limit
	Average (n=5)	% RSD	Average (n=5)	% RSD	
Peak area	48.73	0.7	56.73	0.4	< 2.0 %
Retention time (min.)	4.95	0.3	6.15	0.3	< 2.0 %
Tailing factor	0.94	1.2	0.92	0.8	≤ 2.0
Theoretical plate	12841	0.2	13502	0.2	≥ 2000

Table 6 — Results of robustness study

Conditions	RT of CaOD (min.)	RT of uracil (min.)	Theoretical plates	Tailing factor
Flow rate (0.8 mL/min)	6.31	8.03	13077	0.89
Flow rate (1.0 mL/min)	4.83	6.02	12682	0.94
Flow rate (1.2 mL/min)	4.16	5.29	9401	0.92
pH study @ 2.4	5.51	6.12	12859	0.87
pH study @ 2.7	4.95	6.34	10280	0.87
pH study @ 3.0	4.13	5.51	10216	0.89

RT - Retention time

Conclusions

Newly developed RP-HPLC method is appropriate to separate the impurity present in calcium orotate dehydrate bulk drug. The method was developed using an isocratic condition of mobile phase consists of 0.01 M potassium dihydrogen orthophosphate, pH adjusted to 2.7 with orthophosphoric acid and acetonitrile in a ratio of 98:2 v/v. In HPLC analysis, the retention time of uracil was observed at about 6.02 min in both the reference and CaOD sample. The developed analytical method has been achieved superior resolution (6.32) between the impurity and CaOD.It can be concluded from the HPLC chromatograms that the impurity eluted from CaOD sample is uracil. The resultant statistical data along with low RSD values (< 2.0 %) proved that the method is precise, specific, linear, robust and accurate. The developed analytical method was validated according to ICH guidelines and found to be

applicable for the determination of uracil in CaOD drug. The advantages of the reported RP-HPLC method is economical, reproducible, sensitive and simplicity of sample preparation. Hence, this method can be effectively applied for routine quality control analysis for the quantification of impurity in CaOD drug and for impurity profiling of Generic drug calcium orotate.

Supplementary Data

Supplementary Data associated with this article are available in the electronic form at http://www.nopr.niscair.res.in/jinfo/ijca/IJCA_60A(10)1329-1335_SupplData.pdf.

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