

Isolation, purification and characterization of Cardiolipin synthase from *Mycobacterium phlei* {PRIVATE}

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Received July 08, 2013; Accepted July 08, 2013; Published July 17, 2013

Abstract:

It has been observed that mycobacterial species has high content of cardiolipin (CL) in their cell membranes more so pathogenic mycobacteria and in bacteria CL activates polymerases, gyrases by removing the bound ADP. Therefore, in the present study **cardiolipin synthase** (cls) which catalyses the formation of CL was isolated purified and characterized from the cell membrane of *Mycobacterium phlei*. The purified cls obtained from C-18 RP-HPLC column had a molecular weight of 58 kDa with an isoelectric point of 4.5. The enzyme activity (11.5±0.15 μM of CL phosphorous. ml⁻¹ minute⁻¹ for PG as substrate and 14±0.35 μM of CL phosphorous. ml⁻¹ minute⁻¹ for CDP-DG as substrate) was optimal at pH 4.8 and showed K_M values of 55±0.05 μM and 2.56±0.04 μM for phosphatidyl glycerol and CDP-diacylglycerol, respectively, with an absolute requirement of Mg²⁺ and Mn²⁺ ions for its activity however, Ca²⁺ ions inhibited the activity of the cls. The partial amino acid sequence of cls showed significant homology with pgsA3 gene of *M. tuberculosis* and in this organism the CL biosynthesis is very high having three genes coding for PLs biosynthesis therefore, enzymes involved in CL biosynthesis may be an attractive drug target in the development of new antimycobacterial drugs.

Key words: Cardiolipin, Cardiolipin synthase, CDP-diacylglycerol and phosphatidyl glycerol.

Background:

Mycobacteria has high amount of phospholipids in their cell membranes. The mycobacterial phospholipids include cardiolipin (CL), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI) and phosphatidylinositolmannosides (PIMS). Among the phospholipids of mycobacteria, cardiolipin is often the most abundant (30-50%) of total phospholipids present in both pathogenic as well as in saprophytic species of mycobacteria. Virulent strains of mycobacteria contain a higher percentage of phospholipids as compared to the avirulent strains [1, 2]. High content of CL in pathogenic mycobacteria is further, confirmed by the presence of three functional pgsA genes in the *M. tuberculosis* [3]. The past studies have revealed that crude phosphatide fractions of mycobacteria when injected

intraperitoneally into rabbits elicited typical tuberculosis lesions. Antibodies to phospholipids have been demonstrated in patients suffering from tuberculosis. Recent studies have demonstrated that, during infection of macrophages by mycobacteria, phospholipids (PLs) are released from the mycobacterial cell wall within infected macrophages and transported out of this compartment into intracellular vesicles. The release of these PLs may have functions that influence the outcome of mycobacterial infections Owing to their serological, antigenic and physiological properties, phospholipids of mycobacteria are well studied [4, 5].

The cell membranes of *E. coli*, *S. aureus*, *M. lysodeikticus*, *L. plantarum*, *Bacillus stearothermophilus*, *Bacillus firmus*, and

Mycobacterium smegmatis contains CL which is synthesized by Cardiolipin synthase (cls) and the enzyme is located on the cell membrane of these organisms [4, 6-10]. Kornberg and co-workers reported that CL plays an important role in DNA-replication in *E. coli* by displacing the bound ADP from dnaA protein thereby, reactivating the enzyme required for replication, and also activates gyrases in *E. coli*. Thus, mutation in cls gene makes *E. coli* susceptible to novobiocin. A 10 fold increased activity of cls was observed in *E. coli* in stationary growth phase while the activity of other enzymes in phospholipid biosynthesis decreased, accordingly, CL should play a role in survival of the *E. coli* [8, 11]. Presence of large content of CL in the mycobacteria in particular in pathogenic mycobacteria and its physiological importance of CL in the prokaryotes, it is imperative to study the Cardiolipin synthase involved in the biosynthesis of CL in the mycobacteria. In this report, we describe the isolation, purification and physical and chemical properties of cls enzyme isolated from *Mycobacterium phlei*. The molecular weight, isoelectric point, K_M value of the enzyme, the optimal pH for activity, requirement of various divalent cations for its optimal activity, and the partial amino acid sequence of the enzyme are also established.

Methodology:

Bacterial Strains and culturing Methods

Mycobacterium phlei TMC 1548 was cultured in TYE medium containing 0.2% Tween-20 at pH-7.4. The pelleted cells were washed with 50 ml of 50mM Tris-HCl, pH-7.5, and these cells were used for the isolation of cardiolipin synthase [12].

Isolation and Purification of the cardiolipin synthase (cls)

Preparation of Membrane Fraction

The cells of *M. phlei* were sonicated then centrifuged in a ultracentrifuge Beckman L7-35. The pellet was washed with 50mM Tris- HCl, pH 7.5 and was used as the starting material for purification and isolation of Cardiolipin synthase enzyme.

Assay of Cardiolipin synthase activity

The enzyme assay was carried out in 50 μ M of acetate buffer pH 4.8. Each test tube contained 30 μ M of phosphatidyl glycerol (PG), 10 μ M of ATP, 100 μ M of MgCl₂, 80 μ M of MnCl₂, and 0.1 ml of enzyme fraction (crude or pure). For CDP-diacylglycerol pathway 0.4 μ M of CDP-diacylglycerol was used instead of PG. The reaction mixture was incubated at 37°C for 1 hour, and stopped by addition of 2.5 ml methanol. The solution in the tubes was suspended in chloroform-methanol (2:1 v/v) mixture and stirred for 4 hours at room temperature. The homogenate was filtered and the residue was again stirred for 2 hours in chloroform-methanol (2:1 v/v) and vacuum dried at 45°C in rotatory vacuum evaporator. The dry mass was dissolved in a 1 ml of chloroform, and washed with one fifth volume of KCl and left overnight at 4°C. The upper phase was discarded and lower layer was evaporated to dry in a rotatory vacuum evaporator at 45°C. The residue was dissolved in 0.5 ml of chloroform. This sample was now subjected to thin layer chromatography (TLC). To the activated chromatoplates samples were spotted along with blank and developed for 90 minutes in the Chloroform : methanol : ammonia (115:45:7.5, v/v/v). Phospholipid standards were also run alongwith mycobacterial lipid samples for identification. The plates were air dried and sprayed with 50% H₂SO₄ and heated for 15 minutes at 110°C till charred spots appeared. The spot corresponding to the cardiolipin was

identified in all the samples and scrapped into test tubes and used for the estimation of phospholipid phosphorous. Presence of phosphorous in phospholipids was estimated by Marinetti (1962) method [13]. The K_M values of the purified Cls for both CDP-Diacylglycerol (CDP-DG) and Phosphatidyl Glycerol (PG) were determined. The effect of substrate concentration was first determined by varying concentration of PG between 20 to 100 μ moles. The concentration of CDP-DG was kept constant at 0.4 μ moles. The concentration of CDP-DG was varied from 0.2 to 4 μ moles. The concentration of PG was kept constant at 0.5 μ moles. The enzyme activity of cls was expressed as μ M of CL phosphorous. ml⁻¹ minute⁻¹. K_m and V_{max} were calculated by plotting Hanes-Woolf plot ([S/V] vs [S]) [4, 10, 14, 15]. By taking varying concentrations of MgCl₂, CaCl₂ as 50 to 150 μ moles and MnCl₂ as 40 to 150 μ moles, the effect of Mg²⁺, Ca²⁺ and Mn²⁺ ions on enzyme assay was determined as described in earlier section [4, 5, 14, 15]. The *M. phlei* membrane fractions were solubilized in 2M Urea. After solubilization the suspension was dialysed to remove urea [4, 12].

Purification of Cardiolipin synthase

The solubilized fraction was further fractionated using 30% (NH₄)₂SO₄. This was further passed through Sephacryl S-200 column (1 x 90cm) and cls was eluted. The peak fraction thus obtained was further fractionated on Gel permeation column-HPLC [TSK G-3000SW (21.5 x 300mm)]. 5 μ g active fraction of cls was purified on reverse phase C-18 (4.6 x 150x 5 microns) HPLC (Shimadzu) equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted with a linear gradient of acetonitrile containing 0.1% TFA [4, 12, 14]. The cardiolipin synthase purified on Gel permeation HPLC was analysed on 10% SDS-PAGE. The isoelectric point of the purified cardiolipin synthase was determined using a 10% polyacrylamide native gel rods [4, 12, 16]. The UV absorption spectrum of purified cls was scanned on a DU-64 Beckman spectrophotometer.

Protein sequencing of cls

The peak fraction from reverse phase C-18 (4.6 x 150x 5 microns) column was subjected to amino acid analysis. For N-terminal sequence analysis, cls of *M. phlei* 10 μ g in 25 μ l of 10 mM Tris-HCl, pH-7.5 was applied to prospin device (Applied Biosystems) according to manufacturer's protocol. The resulting PVDF blot was washed twice with 20% methanol prior to sequencing in a Blot cartridge (Applied Biosystems) on a model 477 A pulsed liquid protein sequencer equipped with a model 120APTH analyzer (Applied Biosystems) using methods and cycles supplied by the manufacturer. Data was collected and analyzed on a model 610A data analysis system (Applied Biosystems).

Enzymatic digestion and internal Amino acid sequence

20 μ g of cls in 10mM Tris-HCl, pH-7.5 was dried in a speed vac. (Savant, Formingdale, NJ). The resulting residue was taken up in 25 μ l of 8M urea and 0.4M NH₄ HCO₃ and subjected to reduction, alkylation and proteolytic digestion with 0.3 μ g modified sequencing grade trypsin [17]. The resulting digest was separated by reversed phase HPLC on a narrow bore (21.1 x 250mm) Vydac 218TP52 column and guard column (separations group, Hesponia, CA) and eluted at 0.25 ml /min at 35°C utilizing a gradient on a system Gold HPLC equipped with a model 168 diode array detector (Beckman, Fullerton, CA). Solvent A was 0.1 %TFA in water and solvent B was 0.1 %

TFA in acetonitrile. The column effluent was monitored at 210 and 280 nm. Fractions were collected at 30 seconds interval and stored at -20°C. Fractions (200µl) containing tryptic peptides were applied to Biobrene (Applied Biosystems) treated glass fiber filter and dried prior to amino acid sequencing on the previously described sequencer [17].

Results & Discussion:

The 2M urea was observed to be a useful solubilizer of *M. phlei* membranes while maintaining the enzymatic activity of *cls* (Figure 1A). The solubilized membranes of *M. phlei* were used initially to study the synthesis of CL using both CDP-diacylglycerol (CDP-DG) and Phosphatidyl Glycerol (PG) as substrates and the enzyme activity was observed to be 4.5+0.25

µM of CL phosphorous.ml⁻¹ minute⁻¹ and 6.5+0.25 µM of CL phosphorous.ml⁻¹ minute⁻¹ and the optimal activity of the enzyme was achieved at pH 4.8. This urea solubilized fraction was further used in the purification of *cls* the increased enzyme activity was observed with increased purification (Figure 1B) Table 1(see supplementary material). The *cls* enzyme eluted on GPC-HPLC (TSK G-3000 SW) at a retention time of 42 minutes (Figure 1C) was concentrated and fractionated on C-18 RP-HPLC which was eluted as single peak at a retention time of 15 minutes (Figure 1D) gave single band on SDS-PAGE with a molecular weight of 58kDa (Figure 1E) and IEF analysis showed an isoelectric pH 4.5 (Figure 1F) which confirms that *cls* of *Mycobacterium phlei* is an acidic protein.

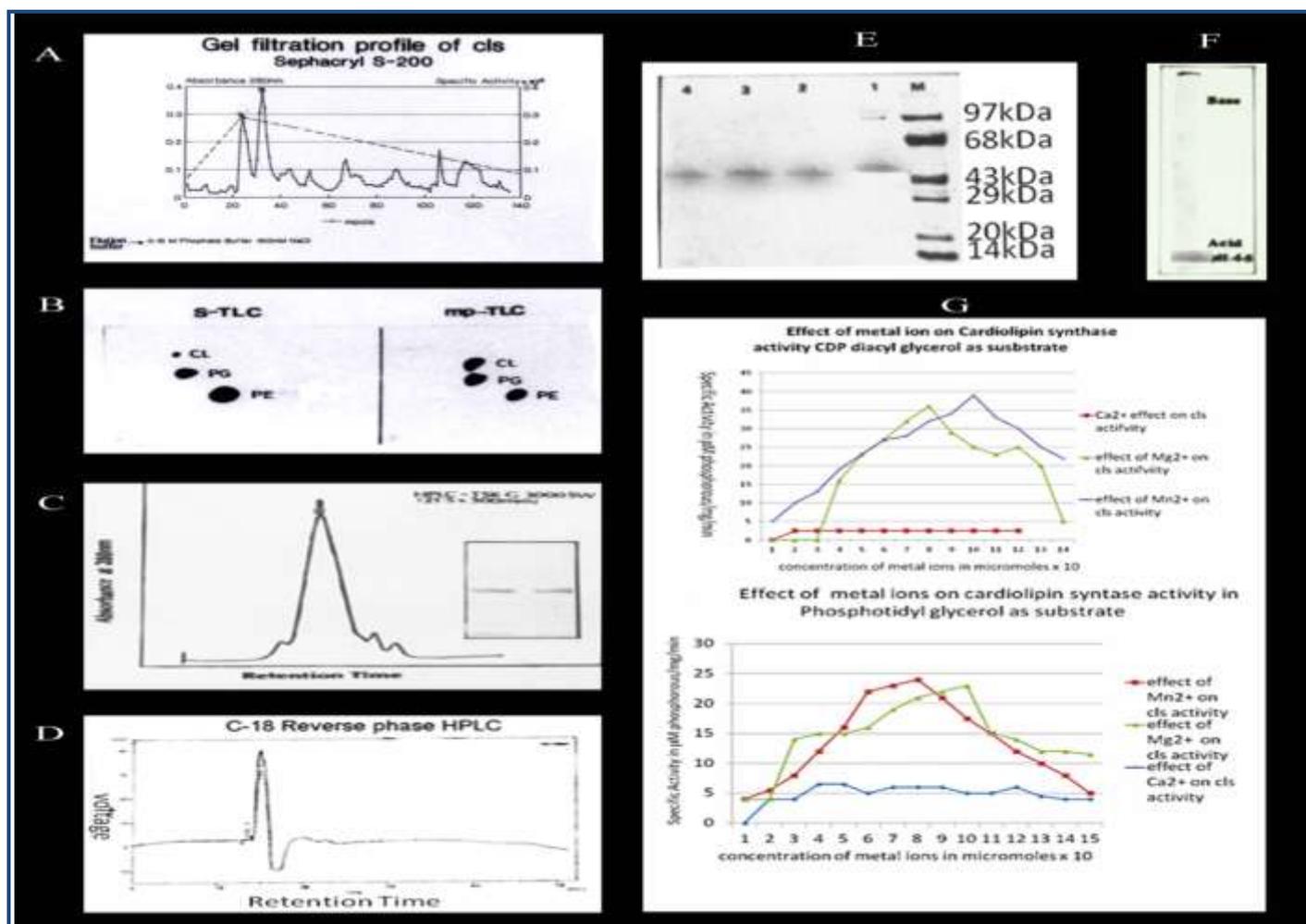


Figure 1: A) Gel filtration profile of cardiolipin synthetase (*cls*) isolated from *Mycobacterium phlei*; B) Thin layer chromatogram of cardiolipin synthesized by the *cls* isolated from *Mycobacterium phlei*. S-TLC: Phospholipids, Phosphatidyl ethanolamine (PE), Phosphatidyl glycerol (PG) and cardiolipin (CL) obtained from sigma, mp-TLC: Phospholipids, PG and CL obtained from the synthesis of CL by *cls* of *M. phlei*; C) HPLC profile of *cls* obtained from Sephacryl S-200 column. 2.5 mg of *cls* purified on Sephacryl S-200 column was applied on HPLC-Gel permeation column [TSK G-3000SW (21.5 x 300 mm)]; D) RP-HPLC profile of *cls* obtained from HPLC-GPC column. 1 mg of *cls* purified from HPLC-GPC column was applied on C-18 RP-HPLC (Waters); E) 10% SDS-PAGE analysis of *cls* purified on HPLC. Lane 1. 5 µg of *cls* purified on Sephacryl S-200, lanes 2 and 3 5 µg of *cls* fractionated on HPLC-GPC lane 4 *cls* purified on C 18 RP HPLC, lane M. molecular size markers obtained from Bangalore genei pvt ltd.; F) Isoelectric focussing of gel pattern of purified *cls*; G) Effects of Mg²⁺, Mn²⁺ and Ca²⁺ on *cls* activity by using CDP-diacyl glycerol and phosphotidyl glycerol as substrates.

The pure *cls* showed an enzyme activity of 11.5+0.15 µM of CL phosphorous.ml⁻¹ minute⁻¹ for PG as substrate and 14+0.35 µM of CL phosphorous.ml⁻¹ minute⁻¹ for CDP-DG as substrate with ISSN 0973-2063 (online) 0973-8894 (print) Bioinformation 9 (13): 690-695 (2013)

absolute requirement for divalent cations with Mg²⁺ and Mn²⁺ in particular however, Ca²⁺ ions completely inactivated the *cls* enzyme (Figure 1G). At an optimal concentration of

phospholipid the K_M values were $55 \pm 0.05 \mu\text{M}$ and $2.56 \pm 0.04 \mu\text{M}$ for PG and CDP-DG, respectively. The cls appeared to be highly thermostable as it retained its 60% activity even at 55°C when kept for one hour. Attempts to detect the amino terminal residue were unsuccessful, indicating that the amino terminal was blocked therefore, the protein was digested with trypsin and the fragments were resolved by C-18 RP-HPLC. The peptide obtained at 72 min had ITDFMDGYIARKYGLKTIAGTILDPLADKL sequence and BLAST-P search analysis indicated that the sequence had resemblance with the cls reported so far (SWISS-PROT data bank with Accession Number-P82260) also the sequence showed significant homology with the pgsA3 (Rv2746c) gene of *M. tuberculosis* H₃₇Rv and pgsA genes of *E. coli* and *Bacillus subtilis*.

The complete *Mycobacterium tuberculosis* genome was recently sequenced and annotated. However, of the 3,924 putative gene products, only a minority are assigned experimentally established physiological functions [3]. Thus, continued analyses of the *M. tuberculosis* proteome are required to fully elucidate the biology of this intracellular pathogen. In this context researcher's all over the world are concentrating and studying various genes in the closely related organisms like *Mycobacterium smegmatis* and *Mycobacterium phlei* in understanding the physiological functions of these genes and implicating their role in *M. tuberculosis*. This would help in understanding quickly the physiological importance of these enzymes/ proteins in the pathogenicity of the organism, further that are required for the formation of successful pathogen [18].

The partial sequence of cardiolipin synthase (cls) of *Mycobacterium phlei* (Acc.No P-82260) showed significant homology with the pgsA3 (Rv2746c) gene of the *M. tuberculosis* H₃₇Rv shows the physiological importance of this enzyme in the mycobacteria which has high amount of cardiolipin in their cell membranes. The high content of cardiolipin (CL) appears to have an important role on the stability of the mycobacteria particularly in the pathogenic mycobacteria, cardiolipin is often the most abundant (30-50%) of total phospholipids. This is further corroborated by the fact that there are three pgsA genes in *M. tuberculosis*, where as only one cls gene is found in *E. coli* [1-4]. Expression of cls is highest in the stationary phase of *E. coli*, since mycobacterial species grows even in low oxygen conditions thus; the requirement of CL is very high in this genus. Thus, probably the CL-biosynthesis is pivotal requirement in the mycobacteria especially in pathogenic mycobacteria [8, 9].

The purified cls showed an absolute requirement for Mg^{2+} and Mn^{2+} ions however, Ca^{2+} ions inactivated cls enzyme. The SDS-PAGE analysis indicated that cls had a molecular weight of 58 kDa while the same cls isolated from *E. coli*, *Bacillus firmus* had a mol. wt of 45 and 57 kDa respectively [7, 9]. The isoelectric point of cls was achieved at pH 4.5 indicating acidic in nature (Figure 1F) which is in agreement that optimum activity of cls was observed at 4.8 pH. Similarly cls of *S. aureus* and *L. plantarum* exhibited optimum activity at pH-4.4 [6, 10]. Temperature dependent analysis showed the cls of *M. phlei* retained its 60 % activity even at 55°C which is consistent with nature of *M. phlei* which can even grow at 55°C . The low K_M values of $2.56 \mu\text{M}$ and $55 \mu\text{M}$ for CDP-DG and PG respectively,

suggests that the cls of *M. phlei* has very high affinity for its substrates. Similar properties were observed for cls present in *E. coli* and in human cardiac cells and in rat liver mitochondrial membranes indicating [8, 9, 14, 15].

It has been observed by Nigou and Besra [5] that phospholipids are released from the mycobacteria within the infected macrophages plays an important role in the outcome of mycobacterial infections. The important factor in understanding the virulence of *M. tuberculosis* is its survival inside the host cell phagosome. The pathogen is able to convert the hostile environment of the phagosome by inhibiting the acidification and phagosome-lysosome fusion [19, 20]. It is very much clear that in CL in bacteria reactivates polymerases and gyrases [11]; it is therefore, possible to predict that the requirement of CL is very pivotal in pathogenic mycobacteria. These findings open up plausible explanations for the presence of high amount of CL in the cell membranes of *M. tuberculosis* for its survival in the macrophages. Therefore; enzymes involved in the biosynthesis of phospholipids may be attractive drug targets in the development of new anti-mycobacterial drugs.

Conclusion:

Cardiolipin synthase which catalyses the formation of cardiolipin, was purified from the membranes of *Mycobacterium phlei*. The purified enzyme showed a molecular weight of 58KD and pI of 4.5. The enzyme showed absolute requirement of Mg^{2+} and Mn^{2+} ions for its activity however, Ca^{2+} ions inhibited the cls activity. The partial amino acid sequence of cls showed significant homology with pgsA3 gene of *M. tuberculosis*. Cardiolipin in bacteria activates polymerases, gyrases and presence of high amount of cardiolipin in the membranes of *M. tuberculosis* possibly explains its survival in the macrophages therefore; enzymes involved in the biosynthesis of CL may be an attractive drug targets in the development of antimycobacterial drugs.

Acknowledgment:

This paper dedicated to late Dr. P.S. MURTHY, who inspired me a lot and ignited many innovative thoughts among scientific entities. I wish his soul to be rest in peace.

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Edited by P Kanguane

Citation: Sarma *et al.* *Bioinformation* 9(13): 690-695 (2013)

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Supplementary material:

Table 1: Purification of Cardiolipin Synthase from *Mycobacterium phlei*

Source of enzyme from <i>M.phlei</i>	Protein concentration(µg/ml)	Enzyme activity Cls PG pathway µg of CL phosphorous/ml/ minute	Enzyme activity Cls CDP-diacylglycerol pathway µg of CL phosphorous/ml/ minute	Specific activity Cls PG pathway µg of CL phosphorous/mg/ minute	Specific activity Cls CDP-diacylglycerol pathway µg of CL phosphorous/mg/ minute	Km (µM) PG	Km (µM) CDP-diacylglycerol
Membrane Fraction solubilized with 2M Urea	29	4.5+0.25	6.5+0.25	16.7+0.5	26.7+0.5	56+0.5	2.65+0.05
30% (NH ₄) ₂ SO ₄ fraction	18	4.95+0.55	7.5+0.35	41.2+1.45	60.6+2.25	55.5+0.25	2.6+0.05
Sephacryl S-200	6.5	10.4+0.25	12.5+0.45	189.4+3.25	249.4+4.25	56+0.5	2.6+0.03
HPLC-GPC TSK-3000SW	1.8	11+0.35	13+0.45	216.21+0.95	286.1+1.05	55+0.05	2.56+0.04
C-18 RP-HPLC	1.6	11.5+0.15	14+0.35	293.21+1.95	350+1.95	55+0.05	2.56+0.04

Values are the mean+ SD from three determinations