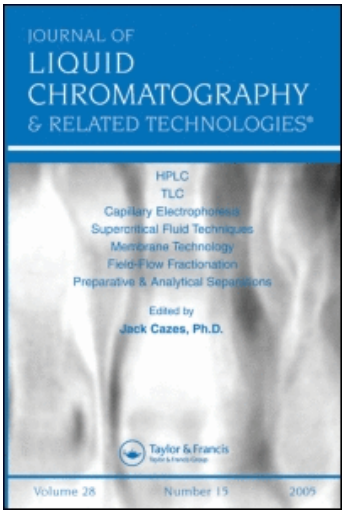

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PREPARATIVE SCALE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND IDENTIFICATION OF CYCLOSPORINE-A FROM AN INDIGENOUS FUNGAL ISOLATE

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ABSTRACT

Extract of fermentation broth of an indigenous fungal isolate of *Tolypocladium sp.*, found to contain Cyclosporine-A (Cy A) has been investigated by spectroscopic and immunological analyses. A preparative scale High Performance Liquid Chromatogrphy (HPLC) for Cy A on an analytical reversed phase HPLC column by interval injection followed by gradient elution has been described. The identity of this preparation was confirmed by matching the spectra with the reference standard using photo diode array detector.

INTRODUCTION

The crude extracts of *Tolypocladium inflatum* Gams were found to have immunosuppressive activity and Cyclosporin-A was

identified later to be the active principle.[1] Extraction of Cyclosporine-A from crude extracts of the fermentation broth was accomplished using laborious and time consuming sequential column chromatographic method[2]. Isolation of Cyclosporine-A (Cy A - VCRC) from an indigenous fungal isolate was reported recently[3] from Vector Control Research Centre, Pondicherry, India. A novel HPLC method is reported here using an analytical reversed phase column for the preparative scale purification of this Cy A - VCRC after the method of Sutfeld.[4] Diode array detection has been used to compare the spectrum of this preparation with the standard Cy-A.

MATERIALS AND METHODS

Chemicals

The fermentation broth of the fungal material in the form of dried powder from an indigenous fungus *Tolypocladium sp.* was received from the Vector Control Research Centre, Pondicherry, India. Cyclosporin A used as standard is Sandimmune from M/S Sandoz Pharmaceuticals. The solvents used for reversed - phase HPLC were Methanol (Lichrosolv) and Acetonitrile (Lichrosolv) from E. Merck, India Ltd. and were used as such. Water was triple distilled in an all glass distillation system and further purified by passing through a NORGANIC Cartridge obtained from M/S Waters Associates (USA). All other solvents used for solvent extraction were of GR grade (E. Merck, India Ltd.).

Instruments

The HPLC equipment comprised of the following : two LC 6A pumps, a Rheodyne 7120 injector, a column oven (CTO - 6A), an

autoinjector, a system controller (SIL-6A) and a data station composed of a C-R3A Chromatopac integrator equipped with a floppy-operated disc system and CRT display, all from M/S Shimadzu, Japan. The detectors used were 1. SPD 6AV (Shimadzu), 2. Rapid Spectral Detector (LKB, Sweden) for the diode array detection operated with an IBM PC/XT equipped with a 20MB hard disc. The spectral and chromatographic data were complemented with the following softwares from M/S LKB, Sweden : a) Wavescan Compare and b) Library Search. These were used for a positive on-line identification of the components identical with authentic sample of Cyclosporine A. The column (25 cm X 4.6 mm ID) was homepacked with Polygosil C18 (5u) obtained from M/S Machery Nagel, Duren, Germany, using a mixture of toluene, dioxane and cyclopentanol as slurry solvents and methanol as the packing solvent. The efficiency of the column observed was 40,000 theoritical plates using a test mixture comprising of benzene, toluene and acenapthene.

Chromatographic conditions

Analytical scale HPLC was performed with the column mentioned above and acetonitrile - water (70:30) at a flow rate of 1 ml per minute. The column was maintained at 75^{o}C. The UV detector was set at 200nm. The "preparative" HPLC was performed with the same equipment for both sample loading and sample elution applying a gradient. The semipure substance previously obtained from various solvent and solid-phase extraction methods was pooled and applied on the column operated with 5% acetonitrile at a flowrate of 1.5 ml/min so as to give a pressure of 230Kg/cm^2 (23MPA). The sample in methanol (100ul) was injected onto the column 54 times at intervals of 1 min. making use of the autoinjector till the pressure abruptly changed to 250Kg/cm^2. The product was recovered by applying a gradient from 10% acetonitrile to 100% with change in flow

rate (to maintain a constant pressure of 230Kg/cm^2) as per the time program given below.

Time (min.)	Event	Percent / Flow rate
0.01	BCONC	10
5.0	BCONC	10
8.0	TFLOW	2.6
30.0	BCONC	80
32.0	TFLOW	2.8
45.0	BCONC	80
60.0	BCONC	100
100.0	BCONC	100
102.0	TFLOW	1.5

The fractions were collected at the points of inflections of the peaks in the gradient elution as per the chromatogram (Fig. 1). The solvents from the fractions were individually reinjected in the analytical mode.

Spectroscopic methods used for confirmation of identity were infrared, ^{1}H NMR, ^{13}C NMR and diode array spectral matches.

Immunological Tests

Immunochemical reactivity of the above product was tested using CYCLO-trac radio immunoassay kit obtained from INCSTAR Corporation, Minnesota, USA. Effects of CY A-VCRC on humoral immune response and delayed hypersensitivity response to sheep red blood cells in mice were tested essentially as reported earlier[5].

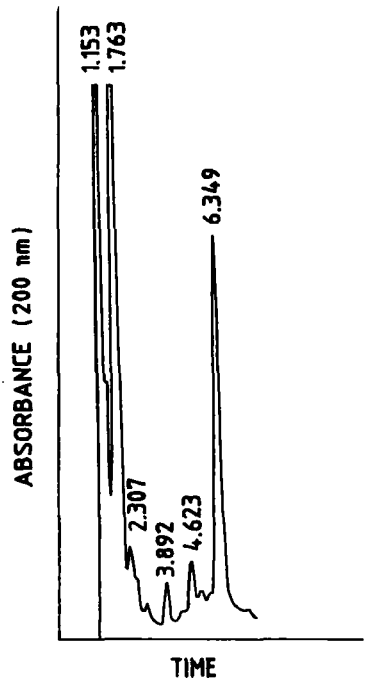

Fig.1 Chromatogram of methanol extract of dried fermentation broth. Peak No. 6 = Cy A. Otherpeaks : unknown. Conditions : Column : Nucleosil C18 (5u) (Machery Nagel), Mobile phase : Acetonitrile - water (70/30); Flow rate : 1ml/min; Temperature : 75°C; Detection : 200 nm (UV).

RESULTS AND DISCUSSION

Preliminary work involved the analysis of the crude extracts of the fermentation broth powder by reveresed-phase HPLC (Fig.1). The peak at 6.54' min. was identified with Cy A extracted from Sandimmune and with the standard Cy A from the CYCLO-Trac radio immuno assay kit. The yield was found to be 0.08 %. The absorbance ratio of Cy A-VCRC as obtained from the diode array detector (DAD) chromatogram also was identical with

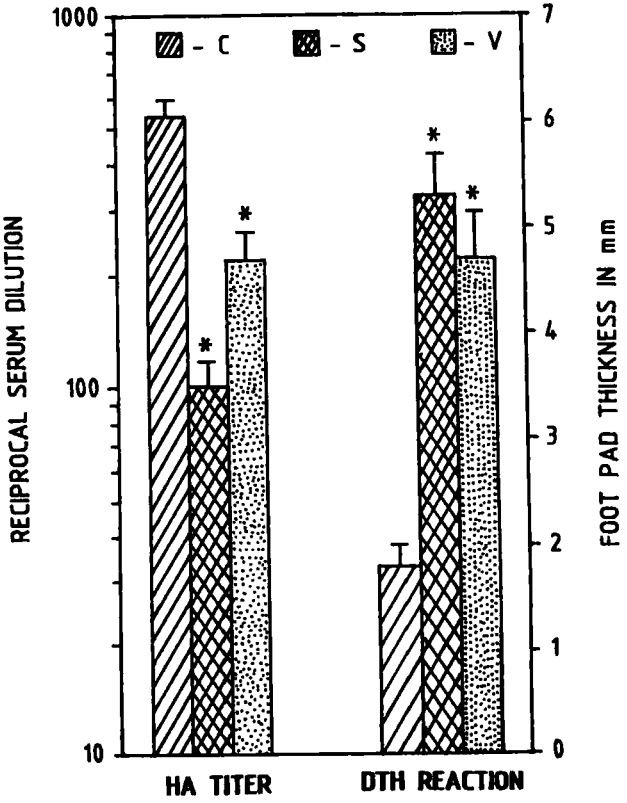

Fig.2 Effect of Cy A-VCRC on humoral and DTH response in Balb/c mice. Cy A-VCRC or Cy A (200mg/kg) injected intra peritonially 48 hr before SRBC immunisation. [control ▨ - C, Cy-A Sandimmune ▩ - S and Cy A-VCRC ▩ - V]. HA titers estimated 8 days after immunisation. DTH reaction was measured 24 hours after injection of 10^9 SRBCs. Results are mean ± SE of at least 5 mice. * - $P < 0.05$.

that of CY A standard (0.456). ^{13}C NMR of the substance obtained from the HPLC fraction was matching with the spectra mentioned in the literature.[2] The immunological investigations revealed the presence of cyclosporin A by radioimmunoassay and there was alteration in the humoral and delayed type hypersensitivity response in mice using the extracts from the fermentation broth (Fig. 2).

Many attempts were made to recover the substance from the mycelia with use of various solvent extractions using Silica, carbon and the carbonaceous packing material from NORGANIC cartridges. In each case HPLC revealed that the product was 98% pure and the DAD spectra matched with that of Cy A standard in the LKB Library with match factor 0.999. Solid matter obtained after removal of the solvent was waxy and tinged yellow. On the other hand manual fraction collection from many HPLC anlysis yielded a colorless solid substance. This was a tedious procedure not suitable for large scale purification.

We then applied the "interval injection" preparative scale HPLC of Sutfeld[4]. About 140mg of the final extracts pooled from several purification methods mentioned earlier was dissolved in methanol, filtered over a 0.45u PTEF membrane filter and autoinjected at the rate of 100ul each injection at intervals of one minute. After about 54 injections, the column pressure abruptly increased to 250 Kg/cm^2. The loading was stopped and after flushing the column with the same mobile phase (5% acetonitrile) as that used for the preparative loading, a gradient elution was carried out as per the time programme described in the materials and methods. Out of the several peaks from manually collected fractions, that between 31.8 to 35 minutes corresponding to 80% acetonitrile in the gradient contained maximum cyclosporine A as per the HPLC analysis by isocratic procedure explained earlier. The DAD Wavescan Compare chromatogram is as shown in Fig. 3 and the spectra in Fig. 4.

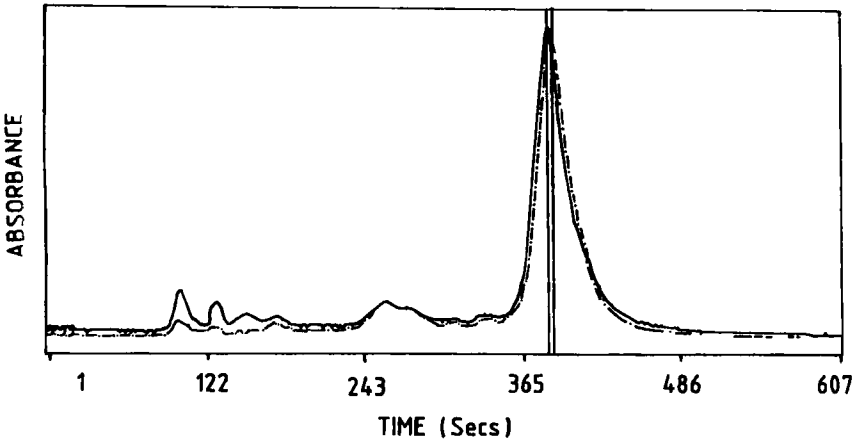

Fig.3 Diode Array chromatograms of Cy A - reference standard (red) and Cy A-VCRC by preparative HPLC. Conditions : Column : Polygosil C18 (5u) and other conditions as mentioned in Fig. 1.

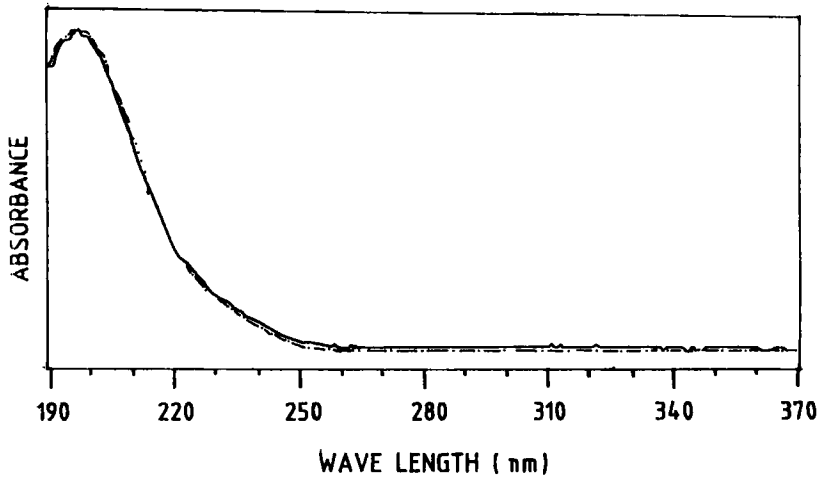

Fig.4 Diode array spectra from the chromatograms in Fig.3.

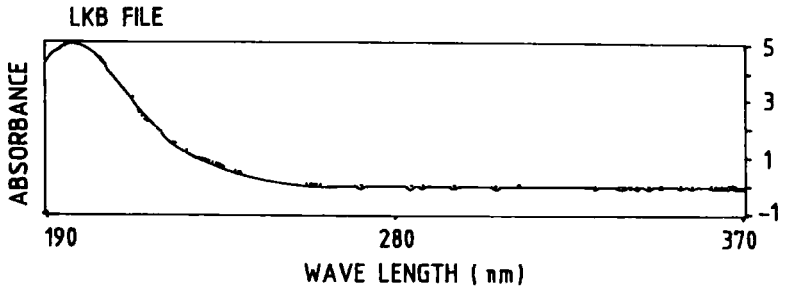

Fig.5 Diode array spectral match with LKB Library Search chromatograms and spectra as in Figs 3 & 4.

The two chromatograms are perfectly overlaid by the software indicating that the standard (dotted lines) and the unknown (continuous line) are indeed same substances. The peak top spectra obtained from the software also match completely (Fig. 5). The Library search software also picked this particular standard out of 27 entries with a match factor of 0.9999.

As mentioned earlier ^{13}C NMR and the radioimmunoassay indicated that the fungal extracts contain CY A, the DAD spectral comparison has further confirmed the identity of Cy A-VCRC. This preparative chromatographic method on an analytical column yielded 33% of the material from the crude extract and the DAD spectum matched almost 100% with that of the reference standard.

ACKNOWLEDGEMENTS

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