REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF METHYLATED AND NON-METHYLATED NUCLEIC ACID BASES

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ABSTRACT

A high performance liquid chromatographic separation method is described for the determination of 5 methyl cytosine and 6 methyl adenine in nucleic acid extract. The bases were separated on a Waters C 18 μ Bondapak column with water: methanol acetic acid system. Effluents were monitored by UV absorption at 254 nm. The bases were estimated by peak heights which are proportional to the amounts of the individual bases. The method is rapid, sensitive, easy to perform and reproducible.

INTRODUCTION

EXAMINATION of the genetic systems in mealybug *Planococcus citri* (Rissi), revealed a few significant genetic differences between paternal and maternal contributions to the embryo whether male or female[1]. It has been noticed that different levels of 5-methyl cytosine (5 MC) and 6-methyl adenine (6 MA) are present in male and female DNA's[2]. This particular observation prompted us to develop a rapid, sensitive and quantitative method using high performance liquid chromatography (HPLC) for the estimation of methylated bases in the presence of other naturally occurring major bases. Although a few reports have appeared in the literature concerning the analysis of nucleic acid bases[3-5], the HPLC separation of 5 MC has not been reported so far. Hence, in this communication a reverse phase method employing Octadecylsilane (ODS) column with isocratic elution is described for the separation of methylated bases particularly 5 MC from other bases.

MATERIALS AND METHODS

All solvents and reagents were of analytical grade. Mobile phases were adjusted to the required pH and passed through Type HA (Millipore) membrane filter. Adenine (A), guanine (G), thymine (T), 5-methylcytosine (5 MC), cytosine (C), ^{6}N-methyl adenine (6 MA), uracil (U), hydroxymethyl cytosine (HMC), 8-azaguanine (aza) and 2-mercapto purine (SHP) were obtained from Sigma (USA). Instrument:- Liquid chromatographic runs were carried out on a Water Associate ALC/GPC 244 series instrument with a Model 6000A constant flow reciprocating diaphragm pump, a septumless injection system (46 K) and a fixed wave length (254 nm) UV flow cell detector (Model 440). A reverse phase C 18 μ Bondapak column (4 mm I.D. $\times$ 30 cm) was used. The mobile phase was pumped at ambient temperature through the column at a flow rate of 0.7 ml/min. The response from the detector was recorded on an Omniscribe recorder at a chart speed of 2.5 min/inch. Mobile phases used were:

System I Water:methanol:acetic acid (96:4:0.25) at pH 2.8–3
System II Water:methanol (96:4) at pH 7
System III Na-acetate (0.01 M):methanol:acetic acid (96:4:0.25) at pH 3.

Isolation and hydrolysis of DNA:- DNA samples were isolated from mealybug according to previously reported procedure[6,2] and were separately dissolved in 0.2 ml of 70% perchloric acid and digested at 100° for 1 hr to obtain the bases in a free form. Before chromatography the hydrolysates were neutralised with 10N KOH.

RESULT AND DISCUSSION

It was found that System I was most suitable for the required separation of methylated bases with respect to the analysis time and resolution power. This system was ideal for the separation of 5 MC from cytosine as compared to System II, since addition of acetic acid reduced trailing of the methylated bases and gave a good resolution between uracil, cytosine, guanine and adenine. Increasing amounts of methanol did not separate uracil from cytosine and guanine from ade-

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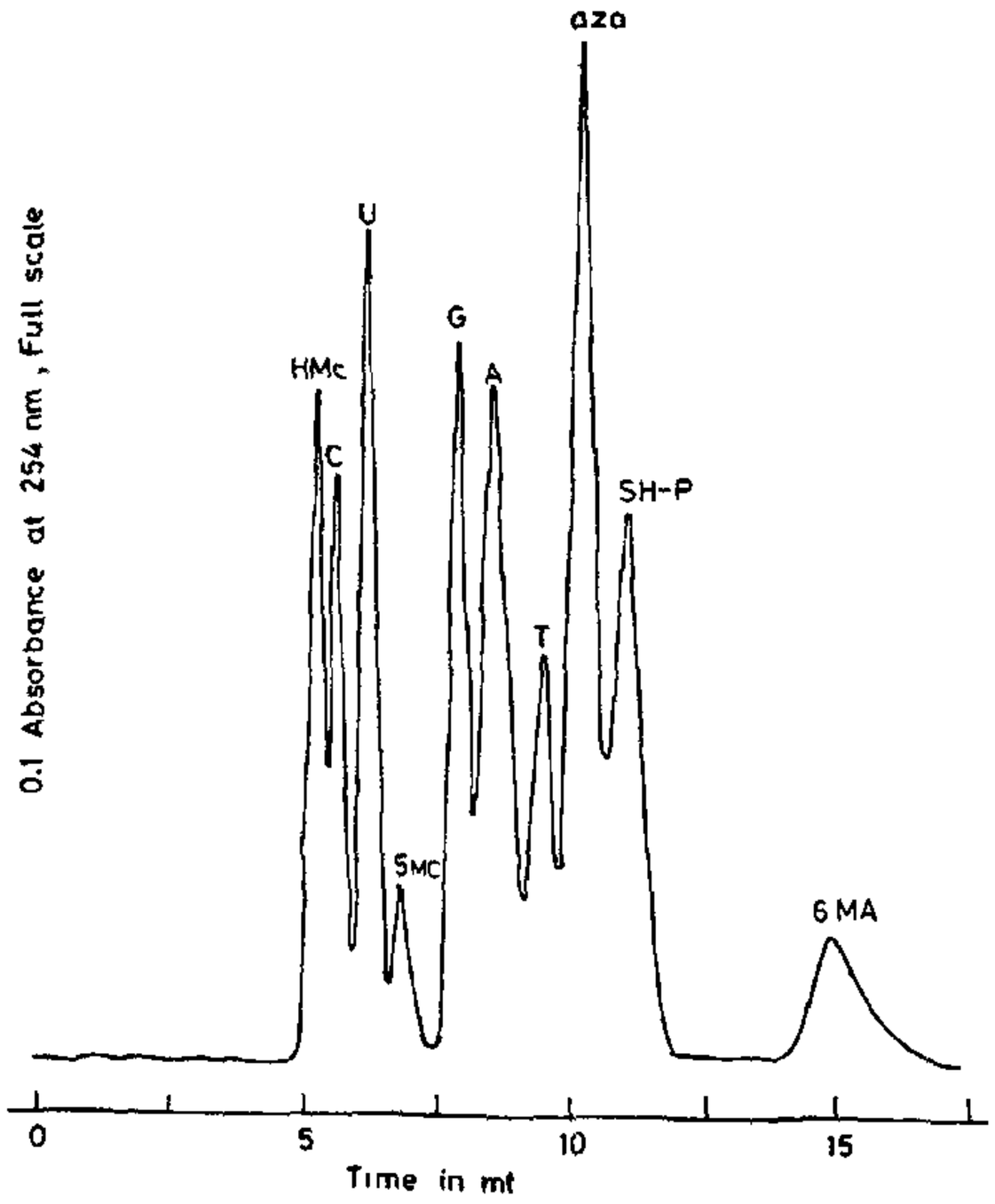

Figure 1. Separation of authentic bases by HPLC on a C 18 μ Bandapak column with water-methanol (96:4) containing 0.25% acetic acid as eluent at a flow rate of 0.7 ml/min.

nine. System III at pH 3 gave a good resolution between cytosine and 5 MC but uracil could not be separated from cytosine. Hence, the mobile phase with water:methanol:acetic acid (System I) was considered ideal to separate the different bases and was routinely used in all experiments. As seen in figure 1 the chromatographic analysis can be completed within 15 min. Under these conditions the lower limits of detection was about 5 ng for 5 MC and 10 ng for 6 MA. The response of the UV detector to known amounts of bases was linear throughout the range (0.02 μg to 0.2 μg at 0.2 auf).

Initial peak identifications were done on the basis of retention time and co-chromatography with standard compounds. In addition, the bases under study were further identified by collecting the column effluent, corresponding to the peaks and determining the optical density ratios at 280/254 nm. The ratio determination at 280/254 nm for the bases used in combination with retention time and co-chromatography with reference compounds has proved to be a valuable aid in identification.

As indicated in figures 2 and 3, this method has been applied very efficiently to detect submicrogram levels of 5 MC and 6 MA in the DNA samples of male and

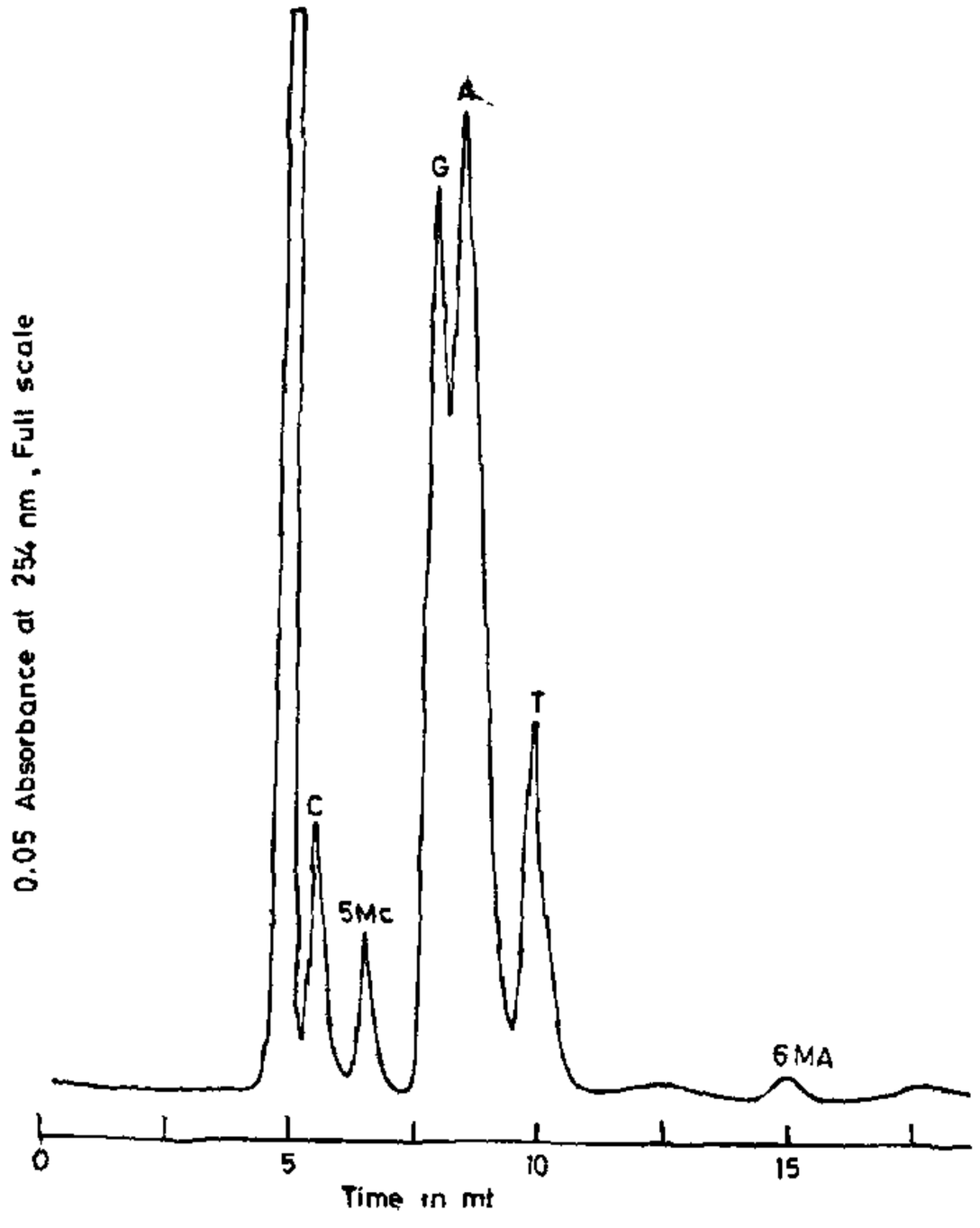

Figure 2. Elution curve of DNA hydrolysate obtained from gravid female mealybug.

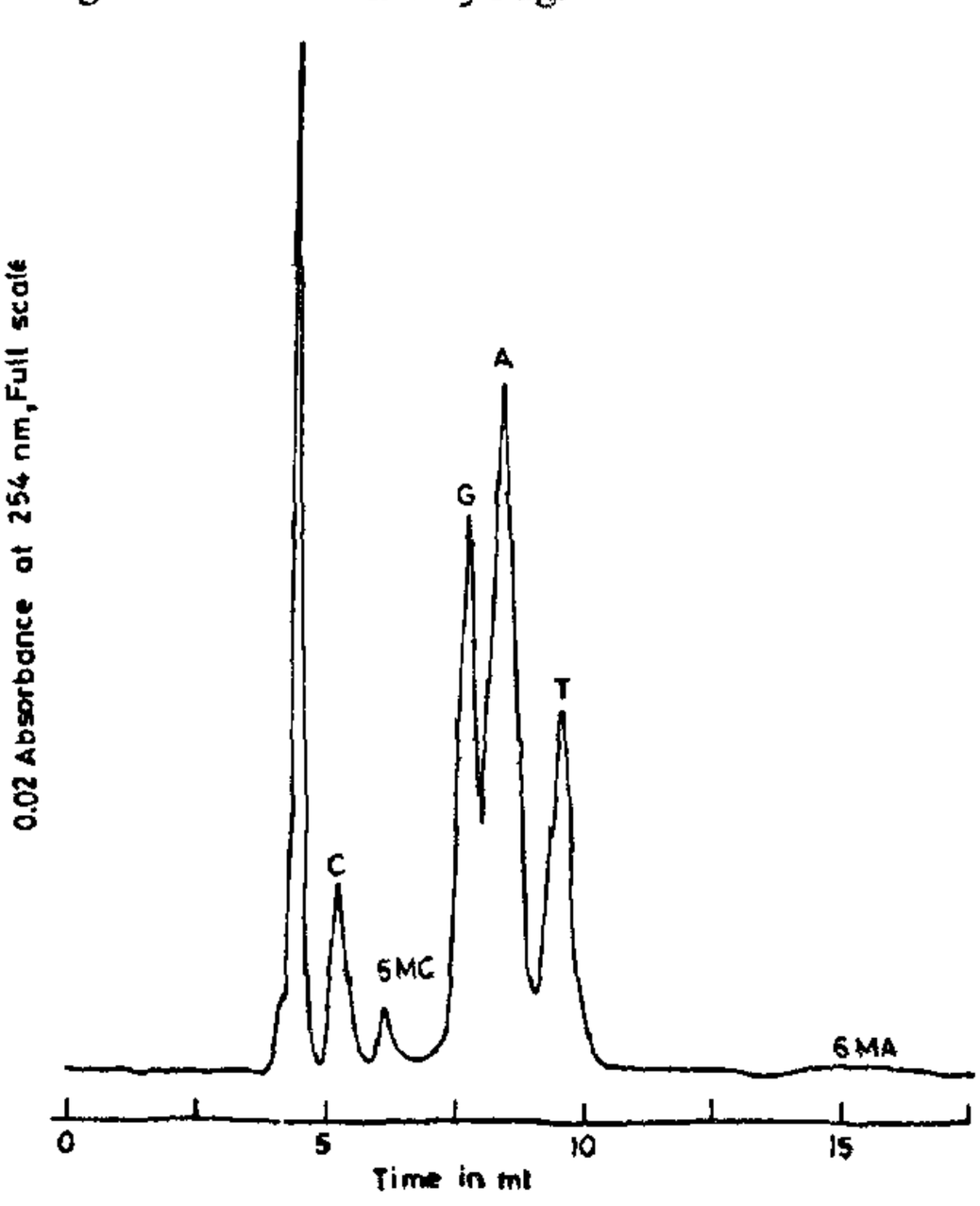

Figure 3. Elution curve of DNA hydrolysate obtained from male mealybug.

female mealybugs. In order to ascertain the authenticity of the peaks corresponding to 6MA and 5MC, DNA hydrolysate was subjected to two dimensional chro-

matography on cellulose plates using water in one direction and n-butanol methanol:water:ammonium hydroxide (60.20.20 l, v/v) in the second direction. After eluting the zones corresponding to standard 6 MA and 5 MC, the extracts were analysed separately HPLC. The retention time and the 280/254 ratio of these compound coincided with that of the authentic compounds. This method is very efficient, sensitive and reproducible for detecting the bases in the complex biological extracts.

ACKNOWLEDGEMENT

Authors are thankful to Professors P. K. Bhattacharyya and H. Sharat Chandra for their helpful discussions.

8 October 1982.

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A SIMPLE AND RELIABLE TECHNIQUE FOR MASS SCALE SERODIAGNOSIS OF HUMAN AMOEBIASIS USING DROP OF BLOOD ON FILTER PAPER

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ABSTRACT

A simple method for the collection, preservation, shipment and testing of minute amounts of dried blood for the diagnosis of amoebiasis is described. A drop of blood obtained from finger puncture and collected on filter paper was extracted in buffered saline. The extracted blood was tested by the indirect haemagglutination (IHA), indirect-fluorescent antibody (IFA) and amoeba immobilization (AI) techniques employing axenic *Entamoeba histolytica* antigen prepared in this Institute. The dried filter paper blood specimens were preserved at room temperature and at 4° C for more than 3 months without detectable changes in antibody response. This technique was evaluated for seroepidemiological survey for amoebiasis among 648 staff members of CDRI classified into 3 different socio-economic groups.

INTRODUCTION

SEROLOGICAL methods for laboratory diagnosis of amoebiasis have been reviewed by Kagan[1]. The test methods used for sero-diagnosis of amoebiasis cases by collecting a drop of blood on filter paper, are fluorescent antibody (FA)[2-4], indirect haemagglutination (IHA)[5-6], and amoeba immobilization (AI)[7-8]. Stool examination for extra-intestinal amoebiasis does not always give positive results. The present study was designed to develop a simple, sensitive and reliable technique, using minute amounts of dried blood from the finger tips which could be used in the detection of *E. histolytica* antibody and, thereby, survey the cases of amoebiasis in selected populations in CDRI.

MATERIALS AND METHODS

Staff members (648) working in Central Drug Research Institute, Lucknow, were surveyed for specific *E. histolytica* antibody in the sera. A drop of blood from the finger on a strip of filter paper (chromatographic 3 mm) was assayed by IHA test method of Krupp[6] who used gluteraldehyde fixed sheep RBC's sensitized with antigen and the IFA method[4]. The AI test followed was same as described by Prakash *et al*[8]. The lyophilized axenic *E. histolytica* antigen (CENTIGEN) prepared in CDRI from axenically grown *E. histolytica* (NIH-200) was used in these tests.

Filter paper strip containing 0.05 ml of blood was eluted in 0.4 ml phosphate-buffer saline (PBS) pH 7.2 and a final serum dilution of 1:16 was obtained. The