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ASSESSING THE SUITABILITY AND EFFICIENCY OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY INSTRUMENT – A NEW APROACH

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ABSTRACT

A unique, cheap, precise and a simple analytical instrument qualification (AIQ) method was developed for a high performance liquid chromatography (HPLC) by using hypoxanthine, a universal nucleobase. This method could be used to determine the suitability of any HPLC that will be used for biomedical, environmental and food sample analysis. The volumetric flow of the mobile phase delivered by the pumps was consistent for given flow rate, with percent relative standard deviation (RSD %) of ± 0.05 % for pump A and B. The hypoxanthine calibration curve for the combined HPLC components in 1% acetonitrile and 0.05 M ammonium phosphate buffer at pH 6, as mobile phase was linear over a concentration range of 4 µM to 20 µM. A correlation coefficient of 0.95 and a sensitivity of 1824.2 AU/µM was obtained. A log-log analysis of the hypoxanthine calibration curve was linear. Column efficiency determined from toluene and biphenyl in 90 : 10 % (v/v) methanol/water mixtures at a flow rate of 1 mL/min. was comparable to the results obtained by the manufacturer. In conclusion, it was determined that the HPLC instrument operated within the manufacturer specified and user approved parameters.

Key words: High performance liquid chromatography (HPLC), Calibration, Column efficiency, Flow rate, Spectrophotometer, Hypoxanthine, Analytical instrument qualification (AIQ).

INTRODUCTION

Assessment of the suitability and efficiency of a high performance liquid chromatography (HPLC) instrument is an important requirement in any analytical separations laboratory. Interestingly, up to now only little information concerning this topic has been discussed and published for HPLC instrument. Analytical instrument qualification

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(AIQ) builds the basis of data quality, which is completed by analytical method validation, system suitability tests and quality control checks. A quick analysis of biological, environmental and chemical samples with HPLC instrument requires a fast, simple and a straight forward system suitability test prior to a formalized validation procedure for specific analytical work. In this method, a simple approach is described in which hypoxanthine (Fig. 1) is used to determine the calibration curve for the combined HPLC components.

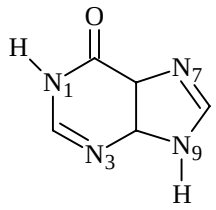

Fig. 1: Chemical structure of hypoxanthine

Hypoxanthine exists primarily in the lactam form, when in aqueous solution; thus, it shows relatively little self association and less vertical stacking characteristics, which tend to make it have less retention time. Analytical instrument qualification test assures that the equipment is adequately designed, maintained, calibrated and tested. Chromatography is a separation technique that utilizes a stationary phase and a mobile phase. The separation process is achieved by a distribution of the analyte between the two phases. The stronger the forces of interaction between the analyte and the mobile phase, the greater the amount of solute that will be held in the mobile phase. Similarly, the stronger the interaction between the analyte and the stationary phase, the greater the amount of solute which will be held by the stationary phase. The distribution coefficient of analyte, defined as the ratio of the concentration of analyte in the stationary phase to the concentration in the mobile phase, indicates the extent to which the analyte interacts with the stationary phase during elution[1]. Subsequently, another parameter which is important in the separation of an analytical matrix is the retention time (t_r) or the capacity factor (k') of the analyte. HPLC separation process is achieved by passing a mobile phase under pressure through a stainless steel column and can be described as a normal phase or a reverse phase separation. The kind of instrument that is used for a particular chromatography separation determines the quality of the analytical method. Further, since the results obtained from HPLC are reproducible for a system with similar specifications, it is significant that easy and precise system suitability method is used prior to analytical work validation. It is important that the components of the HPLC instrument are within acceptable and prescribed limits, without which the system is rendered unsuitable for a meaningful separation work. The main components of an HPLC system are the pump, injector, waste container, column, degasser and the detector.

The pump consists of one or more piston/pump-head assemblies. The pump head is made of 316 stainless steel and has a cavity into which the piston move in and out, which cause the mobile phase solvent to be pushed into the column. The flow rate is determined from a combination of the cavity volume, piston diameter and speed of the piston. The kind of elution method determines the number of pump assemblies to be used in a particular chromatography separation. A pulseless flow of the mobile phase through the HPLC column is important if a meaningful separation is to be achieved. Check valve in the pump head direct mobile phase into the HPLC column. Another important component of the HPLC instrument is the injection valve. This provides a well defined volume of the sample to be introduced into the mobile phase. Sample injection could be manual or automated. In an HPLC instrument testing, the true contribution of the injector alone is often not evaluated, and it is assumed that chromatographic data will really represents injectors precision[2,3]. It is further known that completely filled injection loops provide a better precision than when it is partially filled[2]. Another important component is the HPLC stainless steel column. The dimension of a typical HPLC column is 25 cm x 0.46 cm, and with 5 µm particle size. However other dimensions can be made to suit a particular separation method. The HPLC column life is extended when a guard column is used. The guard column prevents particulate matter in the sample matrix from reaching the column. It is important that 0.01 inch stainless tubing connects guard column to the stainless steel column and detector. The efficiency of the HPLC column is determined from the theoretical number of plates, asymmetric value and resolution of candidate analytical test mixture standards. Retention of analyte within HPLC column depends on functional group, overall polarity, basicity/acidity as well as size and shape of the molecule[4,5]. Finally, the next important component of the HPLC system is the detector. Detector calibration is an important requirement in nearly all investigations involving the quantitative measurements of physico-chemical parameters. A suitable detector determines to a great extent the quality of an analytical separation[6]. The response of the detector used in HPLC must be determined before an analyte's concentration is determined. Further, the dynamic range and the limit of detection of the detector are worth knowing before analysis[7,8]. The response factor of UV-visible detector is determined from the linearity of a calibration curve. The reproducibility of UV-detector response depends on stability of the detector parameters (the voltage and current applied to the lamp, the aging of lamp), the detector wavelength ruggedness and robustness to changes in environmental conditions (temperature, atmospheric pressure, and electromagnetic field), and the stability of chromatographic parameters such as the stability of flow-rate. The linearity of a calibration curve which is a characteristic curve for all components of the system is applicable to detector such as fluorescence, ultraviolet-visible, electrochemical, conductivity, refractive index and mass spectrometry[9-11]. The two important techniques that are used for detector calibrations are the steady state and the pulse injection methods. However, the pulse

injection method is often chosen because the steady state method is highly chemical consuming and not reproducible[12,13]. It is important that the HPLC components (individual components and then the combined components) are properly calibrated to achieve accurate, precise, and reproducible results. In this reverse phase HPLC instrument suitability test, hypoxanthine was used because it gives a lower retention time, shows less vertical stacking and is used as routine standard in our chemical separations laboratory. Further, its physico-chemical properties make it a universal nucleobase.

EXPERIMENTAL

Chemicals and reagents

All the chemicals were of HPLC analytical and American Chemical Society (ACS) grades. Double distilled water was obtained from the Noguchi Memorial Institute for Medical Research. (NMIMR), University of Ghana, Acetone, methanol, toluene and acetonitrile were HPLC grade, obtained from the chemical stores unit of the Chemistry Department at Ghana Atomic Energy Commission, University of Ghana. Biphenyl, ammonium dihydrogen orthophosphate, diammonium hydrogen orthophosphate and hypoxanthine were obtained from the Chemistry and Biochemistry Department, University of Ghana.

Instrumentation

The HPLC system consist of two constant flow dual piston short-stroke pump CE 4100 (Cecil Instrument Limited, Cambridge, England) connected to a dynamic mixture (Cecil Instrument Limited, Cambridge, England) and model 708 Alcott autosampler (Oakbrook parkway, Norcross GA) with a Valco six-port sampling valve. A Hichrom Nucleosil 120-5C18, 250 mm long x 4.6 mm internal diameter (The Markham Centre, Berkshire, England) was connected to a CE 4200 HPLC UV-visible detector (Cecil Instrument Limited, Cambridge, England) which has a cell volume of 8 µL and a path length of 10 mm. The mobile phase was degassed with on-line degasser CE 4020 (Cecil Instrument Limited, Cambridge, England). The acquisition of real time chromatogram was traced with power stream 3.1 software (Cecil Instrument Limited, Cambridge, England) through chromatography system manager CSM unit (Cecil Instrument Limited, Cambridge, England). All separation runs were done at ambient temperature.

Conditioning of HPLC column

Before analysis, the HPLC column was flushed with a degassed 50% (v/v) methanol- water mixture (back pressure 221 ± 5 bars) at a flow rate of 1.0 mL/min for thirty

minutes. This was then followed with degassed doubly distilled water (back pressure 124 ± 5 bar) for another thirty minutes. Finally, the HPLC column was flushed with the mobile phase for thirty minutes to attain equilibration. At the end of days work, the HPLC column was flushed with degassed doubly distilled water (back pressure 124 ± 5 bar) and 50% (v/v) methanol-water mixture (back pressure 221 ± 5 bar) sequentially for thirty minutes each. Lastly, the HPLC column was flushed with 100% absolute methanol (83 ± 5 bar) for thirty minutes and preserved in 100% absolute methanol.

Solvent delivery system

The volume of the mobile phase delivered by each pump was investigated. The flow rate was set to 1 mL/min for ten minutes and the volume of mobile phase (doubly distilled water) was delivered into measuring cylinder measured in three replicates.

Analytical HPLC column

The HPLC column (Nucleosil 120 5µm C18, Hicrom, 25 cm x 0.46 cm) efficiency was determined as specified by the manufacturer. A 90 : 10 (v/v) methanol : water mixture and test mixture standards of 0.21 M toluene and 0.0023 M biphenyl was used for the column evaluation. The UV detector wavelength was 254 nm and a back pressure of 180 bars was obtained. A thirty minute equilibration was attained before analysis. An injection volume of 10 µL of was used and theoretical plates were calculated.

UV-absorbance detector

Approximately 200 µM hypoxanthine stock solution was prepared. The mobile phase consisted of a mixture of 1% acetonitrile and 0.05 M ammonium dihydrogen phosphate buffer at pH 6.0. The stock hypoxanthine solution was serially diluted to the concentrations specified in the calibration curve as shown in Fig. 3. The UV-absorbance wavelength was 254 nm and the injection volume was 10 µL. The wavelength accuracy and drift of the detector was also determined.

RESULTS AND DISCUSSION

The individual and combined components of the HPLC system were tested in this research study. During this instrument suitability test, each of the HPLC pump was used at a time. It was determined that pump A and pump B delivered the exact volume of the mobile phase when the volumetric flow rate of the mobile phase was passed through the column over a set period of time. The delivery of a consistent volume of the mobile phase by the pump is important in determining the quality of an HPLC separation. This is important

because it will allow for the proper interaction between the separating analyte, the mobile phase and the stationary phase. The volumetric flow per minute determined for pump A was 1.024 ± 0.006 mL with percent relative standard deviation (RSD) of ± 0.05 %, whilst that for pump B per minute, it was 1.025 ± 0.005 mL (% RSD ± 0.05). The suitability and efficiency of the volumetric flow of the mobile phase per minute is shown in Table 1.

Table 1: Suitability and efficiency of volumetric flow of mobile phase per minute

Volumetric flow per minute for individual HPLC pumps	
Pump A (mL)	**Pump B (mL)**
1.024	1.025
1.024	1.020
1.025	1.030

Mobile phase: Degassed double distilled water; Set flow rate of pump A and B at 1 mL/min; Average volumetric flow per minute were 1.024 ± 0.0006 (RSD 0.05%) and 1.025 ± 0.005 (RSD 0.05%) for pump A and B, respectively

The calculated flow rate was equivalent to the experimental flow rate of the HPLC pumps. A void volume of 2.45 mL determined for the analytical column was comparable to the manufacturers determined value of 2.50 mL[14]. A flow rate sensitivity less than ± 3.5 x 10^{-3} A for a flow rate change of 1 mL/min to 2mL/min at 300 nm and 2s time constant was obtained. As an initial test to verify on the acquisition of data by the HPLC instrument, 1% acetone was injected into C-18 column and detected at 254 nm with double degassed distilled water as mobile phase. Acetone was used because it is a common organic solvent that is used in the chemical separations laboratory. Further, acetone interacts with water through hydrogen bonding and Van der Waals with the C-18 stationary phase; thus, making it an ideal choice for quick and easy test. A consistent retention time of (t_r = 6.1 minutes) was obtained for this test and this compares very well to acetone retention time under similar experimental conditions[15,16]. However, the peak shape for 1% acetone was not perfectly Gaussian and showed little tailing characteristics. Such peak shape is not ideal in a quality HPLC separations work, and thus suggests that the HPLC column is somewhat deteriorated or there is a heavy load of residues on the column. The column efficiency was further evaluated by repeating the recommended quality control method by the manufacturer. We found that the theoretical number of plates at 50% peak height for the toluene and biphenyl were 2220 (expected : 22800) and 2100 (expected : 20600), respectively. The asymmetric

value for both analytes was 1.7 (expected (both): 0.95), which confirmed the tailing effect obtained for acetone peak. The retention times were 4.04 minutes (expected : 4.37 minutes) for toluene and 4.91 minutes (expected : 5.60 minutes) for biphenyl. A comparison of the column efficiency parameters suggests that the present HPLC column had deteriorated, which consequently showed high back pressures. This normally happens when the HPLC column is not preserved and maintained well. The expected efficiency of the HPLC column was restored after a thorough washing with increasing polarity of elution solvents for one hour. When the 1% acetone was determined again, its peak was a Gaussian. It is important to emphasise here that ghost peaks were not detected as was earlier envisaged for such deterioted columns[17]. Alternatively, the column could have been replaced or repacked with new C-18 particles. The detector response was determined from a calibration curve of hypoxanthine as done previously in literature[18]. The solubility of hypoxanthine was pH dependent and is known to be more soluble at alkaline pHs[19-21]. The hypoxanthine linearity curve over a concentration range of 2.5 µM to 20 µM (Fig. 2) was used to determine the suitability of the combined HPLC instrument[8].

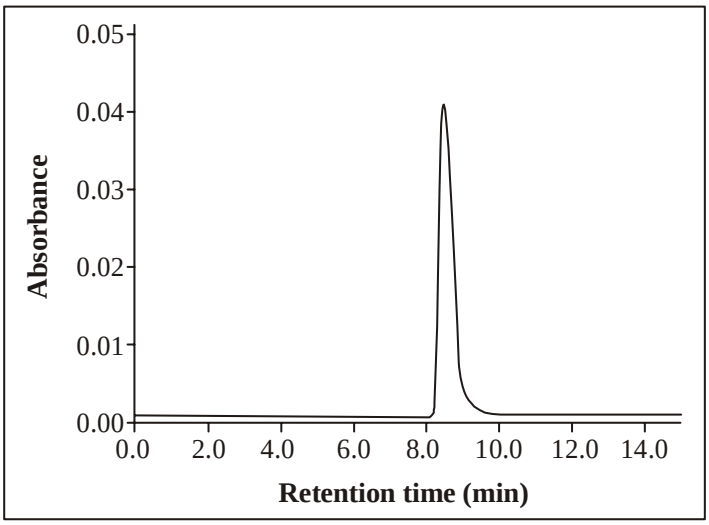

Fig. 2: Typical chromatogram of 20 µM hypoxanthine standard

Column: Hichrom nucleosil 120-5 µm C-18, 250 mm x 4.6 mm; Mobile phase: 1% acetonitrile in 0.05 M ammonium dihydrogen phosphate buffer, pH 6.0. UV-absorbance wavelength: 254 nm (accuracy < 1 nm); Injection volume: 10 µL; Flow rate: 1 mL/min; Retention time: 8.3 minutes.

A detector calibration is an important characteristic in nearly all investigations which involves a quantitative measurement of physico-chemical parameters. The detector drift was

typically less than ± 3 x 10^{-5} absorbance unit per hour at 230 nm. A noise of 0.7 x 10^{-5} absorbance units peak to peak at 220 nm with 2 seconds time constant was obtained. The absolute calibration curve determined for hypoxanthine in this case was achieved according to the IUPAC convention[13]. The peak shape of the hypoxanthine (Fig. 3) from incremental concentration of the standard produced a linear response of the UV detector.

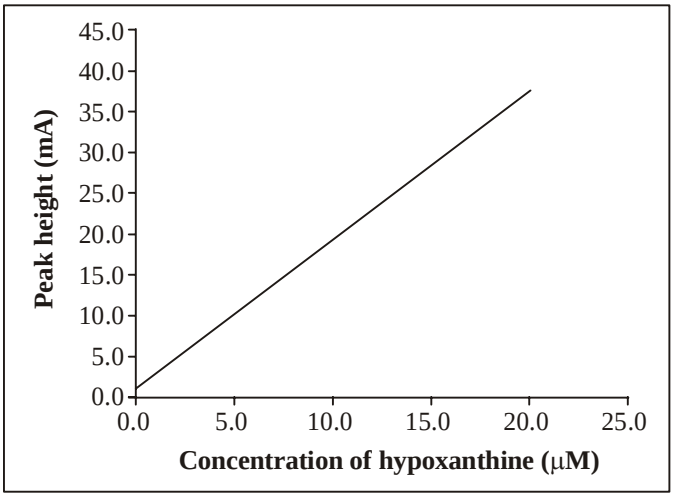

Fig. 3: Calibration graph of hypoxanthine standard

Column: Hichrom nucleosil 120-5C18, 250 mm x 4.6 mm; Mobile phase: 1% acetonitrile in 0.05M NH_4HPO_4 buffer pH 6.0; UV-Wavelength of detection: 254 nm; Hypoxanthine: 20 µM hypoxanthine; Back pressure: 124 bar. Attenuation: 1AUF. Y = 1.8242x + 1.0656, R^2 = 0.95.

The linear equation for the hypoxanthine calibration curve was y = 1.8242x + 1.0656, R^2 = 0.95. As per Beer Lambert's law the extinction coefficient of hypoxanthine was 1824 M^{-1} cm^{-1}. The linear correlation values, sensitivity and intercept for this linear equation was not exactly the same when compared to a linear equation *y* = 19921x -1994.7; R^2 = 0.999 reported in literature under similar conditions[18]. Further, the sensitivity determined from the calibration curve in this experiment was 1824.2 AU/µM whilst that reported in literature was 19921 Aµ/µM. The difference in sensitivities suggests that the UV lamp in the detector was not optimal and maximum light intensity was not attained. A linear least square analysis showed that the standard deviation in slope was 0.16. Although the linear intercepts in both cases suggests a bad background subtraction, the extent of the variation in these values suggest that UV lamp intensity was not accurate. However, the sensitivity of the UV-detector reported in literature[18] was obtained when the UV-lamp in the spectrophotometer was replaced. A response index of 0.99 was obtained from a log of response versus log of

concentration as shown in Fig. 4. This response index was within the established linear response index of 0.98 and 1.02[6].

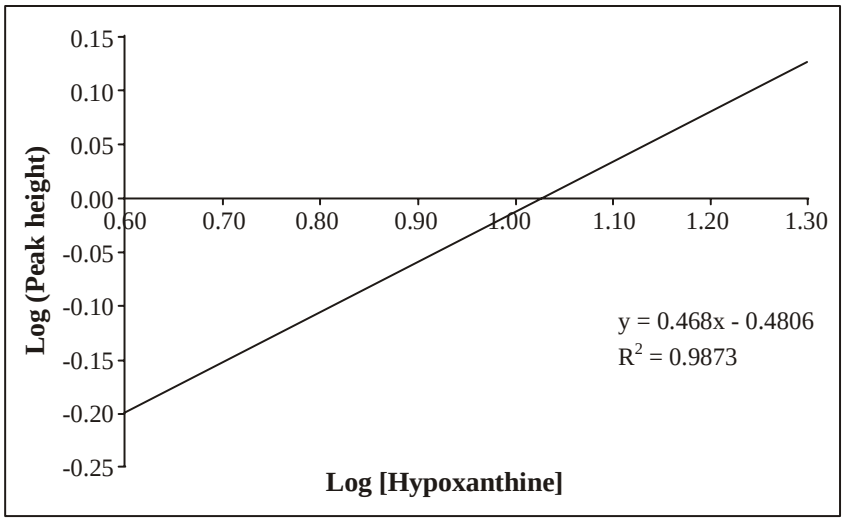

Fig. 4: A Log-Log plot of response versus concentration of hypoxanthine standard. The response factor is 0.99

CONCLUSION

An HPLC instrument was assessed to determine its suitability for environmental, biological, forensic and food analyses. A cheap, simple and readily available mobile phase, sample analytes were used in this validation. It was determined that the dwell volume effect on retention time could be reduced for an isocratic elution method by disconnecting the dynamic mixture from the flow lines. The autosampler and the injector quality of the HPLC instrument were suitable and efficient. The column efficiency and detector suitability of the HPLC instrument was attained with this method. We found that this instrument qualification method for this HPLC instrument was fast, precise and less costly. Hypoxanthine was stable in its pure form and showed less retention time, which makes it ideal analytical standard to be used in any HPLC instrument suitability and efficiency test. This HPLC instrument can therefore be used for both qualitative and quantitative analysis of any given sample matrix.

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