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High performance liquid chromatography (HPLC) Determination of nitrogenous bases Cytosine, Adenine and Guanine by derivatization with 2- hydroxynaphthaldehyde.

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Abstract: Analytical procedure has been developed to determine nitrogenous bases from DNA samples using 2-hydroxynaphthaldeyde (HN) as derivatizing reagent from pharmaceutical and from DNA samples. Three nitrogenous bases adenine, cytosine and guanine were derivatized with (HN) at pH 8 and separated from HPLC column Phenomenexμm C18, 5μm (150x4.6mm id), the determination was done on photodiode array at wavelength 260 nm. The gradient elution was done with methanol, water and acetonitrile CH3CN (58:4:38 volume/volume) with flow rate 1 ml/minutes. The rate of elution of three nitrogenous bases was very rapid within 12 minutes. The method was reiteration with relative standard deviation (RSD) within1, 8-3.7% for each of the compound. The limits of quantification (LoQ) & limits of detection (LoD) were determined at the range of 0.191-0.298μg/ml & 0.063-0.097μg/ml respectively. This method was used for determination of nitrogenous from DNA samples. The results were further confirmed by standard addition technique. The method reports the separation and determination of nitrogenous bases for possible clinical analysis from DNA samples..

Keywords: Adenine, DNA, Guinine, derivatization, High performance liquid chromatography (HPLC)

1. INTRODUCTION

Purine (adenine and guanine) and pyrimidine bases (cytosine and thymine) have great importance in cell metabolism and are known as building blocks of both DNA as well as RNA. Structure and function of products of gene expression – protein are affected Because of changes base in DNA, which causes of inherited disease mostly human cancers (Lindblom *et al.* 1999).Nucleosides are very important for to regulate and modulate the various physiological functions in the central nervous system (CNS) (Fan *et al.*, 2007, 2006). Nucleosides related to various diseases and metabolic disorders are by all vital in biochemical processes. Hence physiological and pharmacological studies of the determination of nucleosides and free bases are very essential (Yang *et al.*, 2007). In physiological liquids including urine (Zheng *et al.*, 2005, blood serum (Yang *et al.*, 2007) and human cerebrospinal fluid (Tavazzi *et al.*, 2005) the component of nucleic acid has been analyzed. They have been separated from medicine, e.g. cordycepssinensis a traditional Chinese medicine (Fan *et al.*, 2007, Yang *et al.*, 2007, Li *et al.*, 20040. The nucleosides have also been analyzed in beer and RNA soft capsule (ShengjieHou and Mingyu 2010). Many biologically relevant compounds (Botta and Bada 2002) are separated for DNA. Using paper chromatography the adenine and guanine as well as triazines (ammeline and melamine) were identified.).

For the determination of DNA several procedures have been developed. Separations are mostly carried out reversed–phase high–performance liquid chromatography (Fan *et al.*, 2007, Gas chromatography- mass spectrometry (Folsome *et al.*, 1973), HPLC with UV Spectroscopy (Vander Velden and Schwartz 1977, Shimoyama *et al.*,1990, Yang *et al.*, 2007, Zheng *et al.*, 2005) normal phase HPLC (Li *et al.*, 2004), Capillary electrophoresis, micellarelectrokinetic capillary chromatograph (Chen *et al.*, 2002), ion pairing chromatography (Tavazzi *et al.*, 2005) ion chromatography (Liu *et al.*, 2008) and liquid chromatography – mass spectrometry (Fan *et al.*,2007, Haung *et al.*, 2003) have developed procedures for quantification of nucleosides. In comparison of other methods HPLC is a simple and widely employed technique in the separation of great variety of samples.

Cytosine is the very essential bases found in DNA and RNA in the four main bases together with adenine, guanine and thymine. Cytosine was hydrolyzed from calf thymus tissues and was discovered by Albrecht Kossel in 1894. Its structure was proposed in 1903 and in the same year was synthesized (and thus confirmed) in the laboratory.

Adenine is one of the most important organic molecules for life. It is basic content of DNA and

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RNA .In DNA it forms base pair with thymine and in RNA with uracil. Adenine occurs in tea and also part of other biologically essential components, e.g ATP, NAD, also vitamin B-12.

Guanine exists both in DNA and RNA, along with adenine and cytosine. There are two tautomeric forms of guanine, the major keto form and rare enol form. Three hydrogen bonds indicate linkage of guanine with cytosine. First time in 1844isolation of guanine was reported from the excreta of sea birds termed as guano used as a fertilizer source. At about fifty years later of its report, Fischer described the structure and told about conversion of uric acid to guanine.

2. MATERIALS AND METHODS

0.5 g/ 500 ml Standard stock solution was prepared in 0.1 MHCl. Methanol, sodium hydroxide, 2-Hydroxynaphthaldehyde (HN), acetonitrile, KCl, HCl (37%), formic acid, acetic acid, sodium acetate, sodium carbonate, sodium bicarbonate, NH_4Cl and ammonia solution were used. Different Buffer systems with different pH were used. CY, AD and GU from the blood serum samples of patients Hepatitis C $^{+}$ve, Tuberculosis meningitis with Hydrocephalus and Brain tumor patients were collected from LUMUS hospital,Jamshoro. The absorbance of the samples was measured on double beam spectrophotometer and chromatographic studies were carried out HPLC (agilent 1100series).

Spectrophotometric method

1 -2 ml solution consisting of CY, AD, and GU was poured to 10 ml flask separately to obtain a final concentration ranges at 25-100 µg/ml. HN reagent 1 ml (0.3% in methanol was added to each of the solution, boric acid – sodium tetra borate pH 8 (1 ml) and analytes were heated on water bath at 800C up to 20 min. The absorption spectrawere noted between 500-250 nm versus the reagent blank after adjusting the final volume to the mark with methanol and. reagent blank was prepared. Same method was followed without the addition of analytes. The reagent blank was prepared.

HPLC Method

0.2-1.0ml of solution containing CY(20-120µg) AD (30-110 µg) and GU (25-100 µg) was taken in the 5ml volumetric flask and added1ml of HN reagent(0.3% in methanol), 1ml of buffer borax buffer solution of pH-8 . The mixture was heated on water bath for 20 min at 80^0C and after cooling at RT; methanol was used to adjust the volume, and 20 µL mixture was injected on the column (column Phenomenex C18, having 5 µm, 150×4.6 mm id). The derivatives were eluted with flow rate of 1 mL per min, with mixture of methanol: acetonitrile: water (58: 4:38) the average peak height was n=4 for each components were measured. The UV detection of samples was measured on photo array at the absorbance 260 nm.

DNA Extraction from Blood (Inorganic Method)

Collection of Sample

The blood samples were collected after consent of the patients, the sample were collected in EDTA tubes and experimental work was carried out at Molecular biology Laboratory, LUMHS, Jamshoro. The DNA was isolated by using acid hydrolysis for the analysis of adenine, Guanine and cytosine. The extraction of DNA was done by organic method and stored at -20 ˚C

Acid Hydrolysis of Extracted DNA.

Aliquot (1 mL) of per chloric acid was added in 100 µL extracted DNA sample in screw capped tube and the tube was heated at water bath (95-100^0C) for 1 hour. Sodium hydroxide (2 M) was used to adjust the pH of the solution to 7 and then filtered.(0.2-0.4 ml) solution was used for HPLC method. From external calibration curve prepared from linear regression equation y = ax+ b. Quantitation of CY, AD and GU was made.Analysis of CY, AD and GU from linear calibration curve with spiked sample.

Sample 100 µg DNA in duplicate after acid hydrolysis were taken. One part was derivatized and injected on the column and the response was measured. Other part was added to 20 µg each of the component CY, AD and GU in 5 ml volumetric flask and after derivatization 20 µL was injected on the column for elution. Response was measuredfrom an increase in response and % recovery of CY, AD and GU from DNA samples was calculated.

3. RESULTS AND DISCUSSIONS

Nitrogenous bases cytosine, guanine and adenine were treated with 2-hydroxynapthaldehyde. These nucleoside bases contain primary amino groups. These synthesized compounds are also known as Schiff bases.

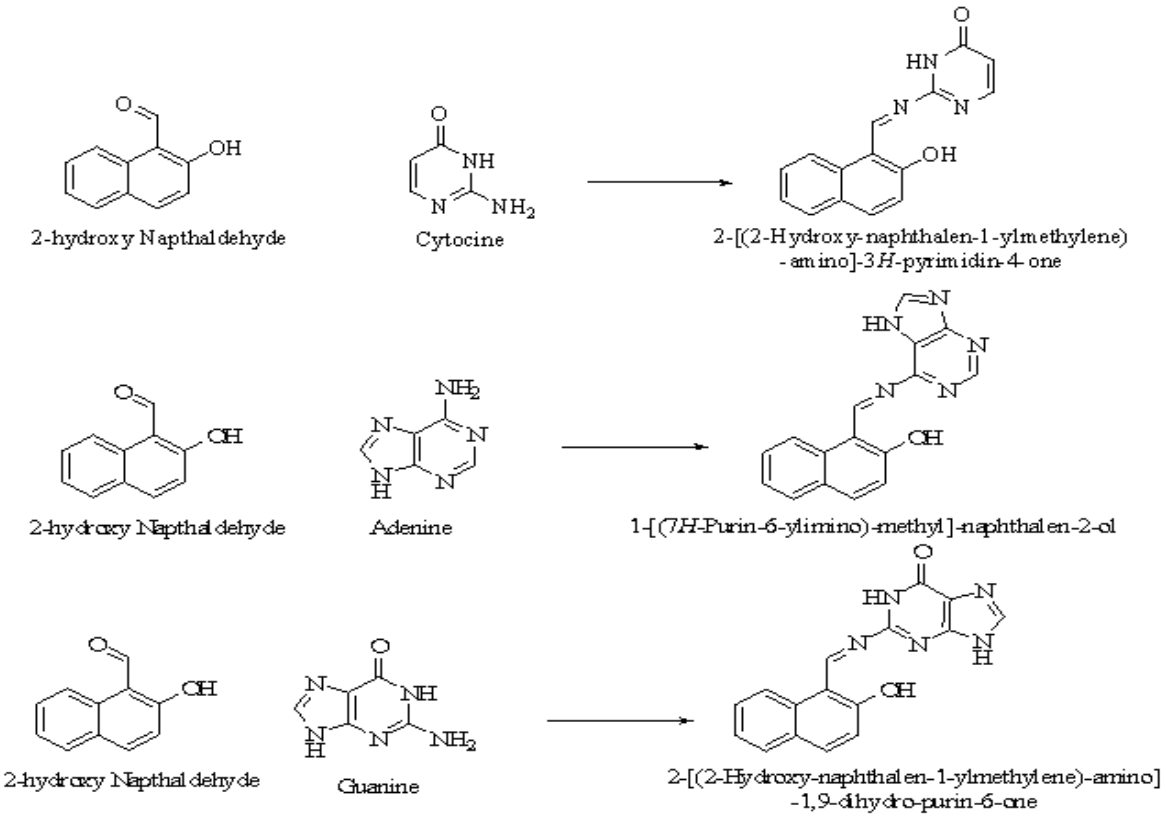

Fig.1 Chemical reaction of HN together with structural formula of CY, AD and GU.

The reaction of HN towards CY, AD, and GU were examined and the products developed yellow color and were initially examined spectrophotometrically. The derivatization conditions were optimized in terms of pH, the amount of derivatizing reagent HN added perseparation, warming time and temperature for derivatization. Condition which indicated maximum absorbance was known as optimal conditions. At unit interval the effect of pH was examined between 3-9.

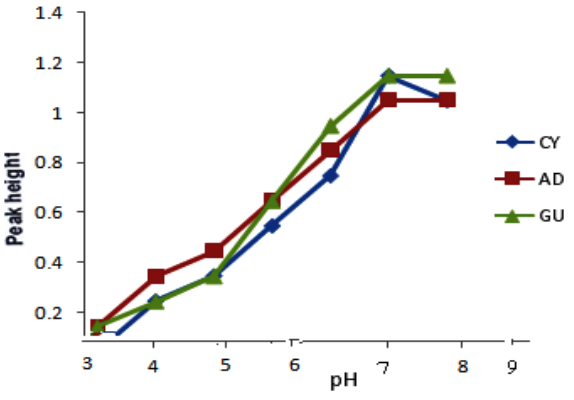

Fig.2. Effect of pH from 3-9 at unit interval on the absorbance of CY, AD and GU as derivatives of HN using heating time 20 min at 80^{0}C.

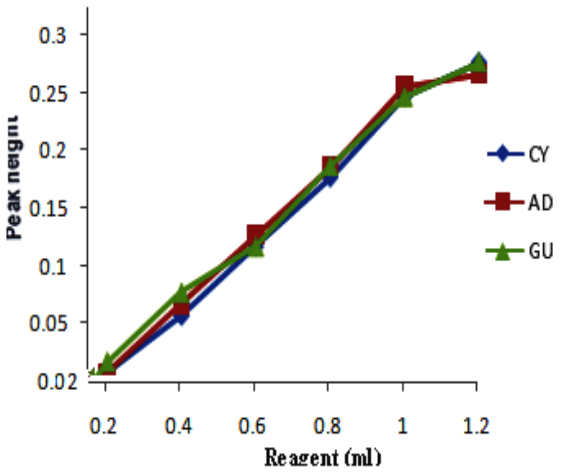

Fig.: 3. Effect of change in amount of reagent with 0.2-1.2 ml (0.3% in methanol) on the absorbance of CY, AD and GU as derivatives of HN using heating time 20 min at 80^{0}C.

The reagent HN (0.3% in methanol) was varied within 0.2-1.2 ml at the interval of 0. 2 ml. Temperature and the warming time and temperature was fluctuated between 20 – 100 ^{0}C as an interval of 20 and 5-30 min at an interval of 5 min. Reactions were monitored at 335 nm.

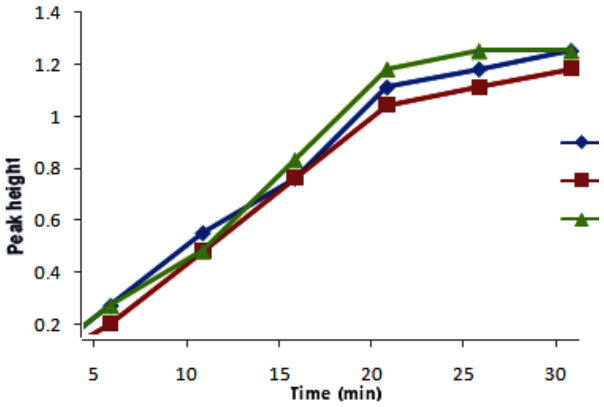

Fig.4. Effect of heating time at 80^{0}C of reagent HN to analytes CY, AD and GU.

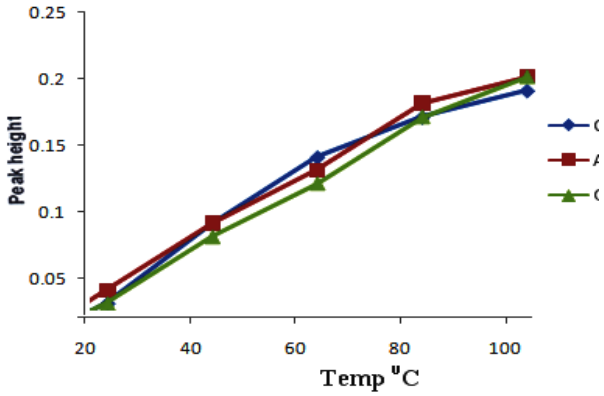

Fig.5. Effect of change in temperature on the absorbance of CY, AD and GU as derivatives of HN using heating time 20 min 20-100 ^{0}Cat an interval of 20^{0}C.

Better absorbance was achieved at pH 7- 8 with a maximum at 8 with sodium tetra borate buffer and was selected. The addition of (HN) required an excess of the reagent to complete the reaction in shorter time. Same response was obtained by addition of 1.0 ml (0.3% in methanol) and above. Thus the addition of 1.0 ml to 5 ml volumetric flask was considered as optimal. Same response was obtained by heating at 80^{0}C for 20 min in water bath and was considered as optimal. HPLC report after derivatization was investigated by intervals of time and no variation in response (average peak area/peak height, (n=4) was observed for CY, AD, and GU investigated up to 18 hours. For the sensitive and simultaneous determination HPLC was checked. All derivatives easily eluted with methanol water and separated from the excess of derivatizing reagent. An attempt was then made to separate CY, AD, and GU as derivative of HN using different solvent systems.

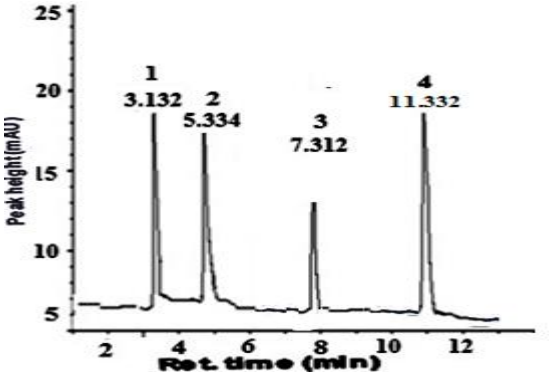

Fig.6 Separation of derivatives of (1) CY(2) AD and(3) GU together with reagent (4) HN from the Column Phenomenex C – 18, 5 µm (150×4.6mm id) with simple gradient elution of methanol – acetonitrile – water (58: 4: 38) with a flow rate of 1 ml/ min.

Maximum separation was obtained from the Phenomenex C-18 column when eluted iso critically with methanol– acetonitrile -water (58:4: 38 v/v/v) with a flow rate of 1ml/min and ultraviolet detection was measured at 260 nm. The elution time for mixture of derivatives was within 12 min. The capacity factor (k') was calculated to 2.80, 3.65 and 4.63 for CY, AD, and GU as derivatives of HN. The separation was repeatable with (RSDs) was within 3.7 % (n=5).

Table 1: Quantitative HPLC data of derivatives CY, AD and GU with reagent HN

S. No	Name of compound	Calibration range µg/ml	Coefficient of Determination	Linear regression equation	Detection limits µg/ml	Limits of quantification µg/ml
1	CY	1-25	0.996	Y=7.434x+0.071	0.082	0.249
2	AD	1-24	0.999	Y=18.952x+0.268	0.097	0.0191
3	GU	1-20	0.993	Y=24.955x+0.229	0.097	0.0298

Table 2: Analysis of acid hydrolyzed DNA of human blood for CY, AD and GU concentration µg/ml together with reagent HN

Purine& Pyrimidine Bases	Blood S.1	Blood S.2	Blood S.3	Blood S.4	Blood S.5
CY *Sample	1.079 (1.80) 1.089 (2.5)	1.075 (2.9) 1.085 (1.6)	1.071 (2.2) 1.075 (0.7)	1.079 (0.4) 1.083 (2.7)	1.075 (0.5) 1.069 (3.0)
AD *Sample	1.089 (3.1) 1.087 (2.3)	1.068 (2.6) 1.061 (1.3)	2.070 (4.8) 2.076 (1.6)	1.087 (3.7) 1.088 (1.5)	1.078 (2.6) 1.067 (3.5)
GU *Sample	1.086 (2.6) 1.079 (1.9)	1.072 (2.7) 1.076 (1.3)	1.073 (2.5) 1.081 (0.5)	1.076 (0.8) 1.086 (1.3)	1.073 (2.4) 1.075 (3.4)

The values given in parenthesis represents %RSD, Cytosine (CY) Adenine (AD) Guanine (GU) S.1 sample 1 *Sample = Spiked sample.

Quantification:

The linear calibration curves were achieved by plotting average peak height/peak area (n=4) against concentration within 1-24 µg /ml with a coefficient of determination (R2) between 0. 993- 0. 999. limits (LoDs) and (LoQs) measured as signal- noise ratio (3: 1) and (10: 1) ranged from 0. 163 - 0.197 µg/ml and 0.471-0.592µg/ml respectively. The dervitization, separation and quantitation was repeatable in condition to retention time and average peak height / peak area (n = 4) inter (n = 5) and intra (n=4) day when carried out by same operation on the same day and different days and standard deviation were obtained between 1.8, 2.2%, 2.5 -3.2% and 3.7- 4 % respectively. Additives of pharmaceutical preparations methylparabin, propylparabin, manitol, gum acacia, sodium lacuryl sulfate, glactose, lactose, glucose, , sucrose, sodium chloride and methyl hydroxyethyl cellulose at least twice the concentration of CY, AD , and GU were added and the analyses were carried out following HPLC procedures. Result obtained was compared with analytes standards. The additives did not interfere the analysis with relative deviation within 3.7 %. Sample analysis was made on the bases of comparison of retention times with thestandards. The quantitation was made from external calibrations based on least squares regression equation **(Table 1).** The analytes CY, AD, and GU are available blood DNA after acid hydrolysis. The method was applied for their analysis for the possible determination. The analysis was carried out after extraction of the DNA from blood samples as reported by Ali et al (2013) following the inorganic method Grimberg et al. (1989). The isolated DNA was acid hydrolyzed with per chloric acid. The analytes CY, AD, and GU were then analyzed following analytical procedure. The results ofanalysis are shown in **(Table, 2)** with RSDs within 3.9%. The blood serum samples were also analyzed by standard addition. Results of analysis were good agreed with that of external calibration curves and % of recovery of CY, AD, and GU in blood serum was calculated 98, 94, and 102% with RSDs 1.9, 2.6, and 3.8% respectively. Comparison between the results of present methods with reported methods.

The results for Cytosine (C) agreed with that of (Xia *et al*. 2011) and also for Cytosine (CY), Thymine (TH) and Adenine (AD) with (Xia *et al.*2009) for human plasma samples. Our results for plants DNA nucleobases also agreed with the reported values by (Zhao *et al.*2013).

Table: 3. Comparative studies of the reported methods used for the determination of Purine & Pyrimidine bases with present methods.

Method	Compound Determined	Derivatizing Reagent	Calibration Range	Limits of detection	Analysis Time	Application	Reference
C.E	U,Crt,Th, Ad,Ur and Gu	-	5-500 µmol/L	0.84-4.25 µmol/L	20 min	Urine Samples	T. Adam et al. (1999).
GC MSMS	Ur,Th, Dihydrothymine	tert-Butyl dimethyl silyl	25-2500 pmol/L	0.80-8.0 pmol/L	12 min	Urine Samples	U. Hofmann et al. (2003),
HPLC	Cy,Gu,Th, Ad,5-Methylcytosine	-	0.1-10 µM/mL	26.3-162.1 nM/mL	15 min	(Calf Thymus and Fish Sperm)	T.A. Ivandini et al. (2007)
HILIC and ESI-MS	Th,U,Ad, Cy,Gu and Xanthine	-	0.001-5.00 µg/mL	0.15-10.0 ng/mL	60 min	NaturalCordyceps	H-Q Zhao etal. (2013)
HILIC	Cy,U,Gu, and Xanthine	-	0.0025-1.816 µg/mL	0.07-30.49 ng/mL	110 min	Geosaurus and Leech	P. Chen et al. (2011)
HPLC	Ad,Cy,Th and Gu	HN	1-24 µg/ml	0.163- 0.197 µg/ml	12 min	Human Blood & Plant DNA (Cotton, Jasmine)	Present method

The developed HPLC method was also compared with reported methods in terms of calibration range, LOD, analysis time and applications. Our developed procedure is sensitive then the reported methods (Werner *et al.*,1987)

(Adam *et al.,* 1999), (Hofmanna *et al.,* 2003), (Ivandini *et al.,* 2007), (Zhao *et al.,* 2013), (Chen *et al.,* 2011) but indicates comparable of shorter analysis time and broader calibration range for all the compounds analyzed.

This work aims to develop a simple and sensitive HPLC procedure for the separation of nucleosides cytosine, adenine and guanine. This work also checked HN reagent for the HPLC coupled with commonly available UV detection for the determination of CY, AD and GU from DNA samples. The conditions are optimized for pre – column derivatization, HPLC separation, quantitative determination and validation of analytical method.

4. CONCLUSION

For the separation of biological active compoundsCY, AD, and GU from blood serum an analytical method has been developed by HPLC using pre-column derivatization with 2-hydroxynaphthaldehyde. The analysis agreed with reported values with % recovery of analytes from acid hydrolyzed DNA samples within 96- 106 % with RSDs with 1.8 -3.7%.

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