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QUALITATIVE AND QUANTITATIVE ANALYSIS OF VARIOUS CONSTITUENTS OF VACCINES BY USING ANALYTICAL TECHNIQUES

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ABSTRACT

The present study deals with the various analytical techniques like ultra-violet spectrophotometer, high performance liquid chromatography and AxSYM system for the quality control of various vaccines. Ultra-violet spectrophotometer was used to analysis the thiomersal content in Diphtheria tetanus pertussis (DTP) vaccine 0.0107%, protein content in Rabies vaccine 0.657%, and phenol content in Diphtheria tetanus pertussis vaccine 0.230% respectively. High performance liquid chromatography was used to determine the molecular size distribution of the meningococcal vaccine 97.50% and the AxSYM system was used to estimate the in-vitro potency assay of hepatitis B 0.9769%. Formalin content in Diphtheria tetanus pertussis (DTP) vaccine 2.0% was estimated by qualitative analysis. It was confirmed that results were found after quantitative and qualitative analyzing the constituents of vaccine samples with the analytical techniques employed in the present study accurate, efficacious and rapid rather than the other previous method.

Keywords: Analytical techniques, AxSYM system, HPLC, UV-spectrophotometer, Vaccine.

INTRODUCTION

Analytical chemistry is the science of making quantitative measurements and it is the chemistry discipline concerned with the chemical composition of materials. Analytical chemistry mainly divided into two group‟s qualitative analysis and quantitative analysis (Helmenstin, AM, *et.al*, 2009). Spectroscopy (quantitative analysis) was a new field of study for the thiomersal (preservative), protein (binding agent), phenol (preservative) content in the vaccine (Plotkin, SA, 2006). Indian pharmacopoeia described assay method using colorimetric titration and simpler spectrophotometer. But UV-spectrophotometer method of analysis is more economic and simpler (IP, 2007). Under computer controlled instrumentation, UV-spectrophotometer playing a very important role in the analysis of vaccine samples (Greenlief, P, 2001).

High-performance liquid chromatography is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture. It is basically a

highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced under high pressures up to 400 atmospheres to makes it much faster (Snape, MD and AJ Pollard, 2005). The molecular size of polysaccharide vaccines is an important pysico-chemical parameter which correlates with immunogenicity (Lloyd, RS and JW Dolan, 2007). Many other method used for the determination of size distribution to the polysaccharides vaccine i.e gel filtration and ion exchange chromatography but the latest model-560 HPLC is the most important than the other perivious method because it shows the sharp peak , have high resolution power, superior refractive index detector and rapid monitoring techniques (Richmond P *et.al*, 1999).

The AxSYM is an immunochemical automated analyzer made by Abbott Laboratories. It is used for serology tests and therapeutic drug monitoring, and uses antibodies to alter the deflection of polarized light. It can also be used to monitor hormone level and some cardiac markers such as troponin (Jilg, W *et.al*, 1984). The main purpose of this system to estimate the *in-vitro* potency assay of hepatitis B vaccine. Appearance and use blood samples and reagents are placed in separate carousels on the right of the machine. This instrument is used in medical laboratories by trained medical personnel. It can process about 100 samples an hour (Shipchandler, MT and Moore, EG, 1995). Potency of the vaccine determined by two method *in-vivo* and other by *in-vitro* or analytical technique i.e. AxSYM system. The *in-vivo* method is a time-consuming and labor intensive process. The animal ethics committee makes the rule to avoiding the animal death. *In-vivo* studies are very pain full, So that the new method established for the estimation of hepatitis B potency. This analytical technique is most important, accurate, precious, efficient and specific than the *in-vivo* and previous potency assay study (Karimzadeh, H *et.al*, 2010).

The main focus of the present study, an attempt was made to perform the qualitative and quantitative analysis of various constituents of vaccines using analytical techniques.

MATERIALS AND METHODS

Chemicals

Formaldehyde, phenyhydrazine hydrochloride, potassiumferrocyanide, formalin standard, hydrochloride, nitric acid, aqueous solution of thiomersal , solution of ammonium acetate, dithizone, chloroform, stock standard: bovine serum albumin of concentration 5 mg/ml, working standard, biuret reagent, 4-aminoantipyrine, borate buffer, standard phenol solution concentration 0.25%, sodium dihydrogen phosphate, sodium chloride, sodium azide, dextran, acetone, MQW (Milli Q Water), Anti-HBs (Mouse, Monoclonal, IgM) coated microparticles in phosphate buffer with protein stabilizers, minimum concentration 0.15% preservative: sodium azide, anti-biotin (Rabbit), alkaline phosphatase conjugates in tris buffer with (Rabbit) IgG, minimum concentration 0.03µg/ml preservative: sodium azide, biotinylated anti-HBs (Goat, IgG) in tris buffer containing animal sera (Goat, Calf, Rabbit, Mouse), preservative: sodium azide, Quinolone, PBS + 2% BSA. The entire chemicals are obtained from the Central Research Institute, Kasauli, distt. Solan, Himachal Pradesh.

Instrument

UV-spectrophotometer manufacturer by Jainco Inks & Chemicals Karnataka, HPLC model-560 with PDA and RI detector manufacturer by central drug house Pvt. Ltd. New Delhi, , AxSYM system manufacturer by Abbott Laboratories , solvent filtration unit with receiving flask, Laboratory Vortex Mixer, Bio Safety Cabinet, pipettes and tips obtained from E- Merck Pvt. Ltd. Mumbai.

Vaccine

DTP, Rabies, Typhoid, Meningococcal, Hepatitis B. The entire vaccines are authenticated from The Central Research Institute Kasauli, distt. Solan, Himachal Pradesh. All the vaccines stored at the temperature 2-8°C.

Estimation of Formalin Content by Qualitative Method

Formalin or formaldehyde is used as a detoxification agent who converts the toxin of the antigen into toxoid (Plotkin, SA, 2006). Estimation of formalin content test performed on the DTP, vaccine by using qualitative analysis. In this method formalin is added to the sample as a detoxification agent who converts the toxin of the antigen into toxoid and the left free formalin is to be tested. Phenyl hydrazine hydrochloride from a complex with the free formalin and the potassium ferrocyanide acts as an indicator which gives color different shades on the addition of conc. hydrochloride.

Estimation of Thiomersal Content by Chemical Method Using UV-Spectrophotometer

Thiomersal is an organomercurial salt complex of sodium ethyl mercuriothiosalicylate containing ethylmercury that has been widely used as a vaccine preservative (COX, NH and A Forsyth, 1988). Estimation of thiomersal content test performed on the DTP vaccine by using UV Spectrophotometer. In this method Separating funnels were washed with concentrated nitric acid and rinsed with tap and distilled water. Two aliquots of a standard 0.01% aqueous solution of thiomersal (0.5 and 1.0 ml) and two samples of the test solution (1ml) were added to individual separatory funnels. The volume was adjusted to 10 ml with 1% solution of ammonium acetate at pH 6.0. Ten milliliters of a 1 in 10 dilution of a fresh solution of 0.01% dithizone in chloroform was added to each funnel. The contents were shaken vigorously for 45 seconds. The chloroform layer was separated carefully and spectrophotometer was set at 490 nm using the diluted dithizone solution and a scan were taken of the test solution from 470- 520 nm.The transmission at 520 nm was plotted against the thiomersal concentration of the standards on semi- logarithmic paper and the concentration of thiomersal in the sample was determined. The thiomersal content can be calculated by following calculation.

$$\text{Thiomersal content} = \frac{\text{O.D. of Test}}{\text{O.D. of Standard}} \times \text{Conc. of Standard Thiomersal}$$

Protein Estimation by Biuret Method Using UV-Spectrophotometer

Estimation of the protein content test performed on the Rabies vaccine by biuret method using UV spectrophotometer (Stoscheck, CM, 1990). Under alkaline conditions, substances containing two or more peptide bonds from a purple complex with copper salts in a reagent. The concentration of more protein in the sample is determined by the concentration of peptide bonds. More the number of peptide bonds more are the concentration of the sample (Smith, PK, *et al.*, 1985).The spectrophotometer was switched on 15 minutes prior to performing the test 1ml of distill water were added in a test-tube marked as „Blank‟. Various concentration (4mg/ml, 3mg/ml, 2mg/ml and 1mg/ml) of the standard were prepared from the stock standard solution (5mg/ml) and take 1ml each of various standard concentration in a test-tube marked as „Standard‟. Prepare 1:10 dilution of the sample (0.9 ml of distilled water and 0.1 ml of vaccine sample) were prepared and taken the 1ml each of the samples in test-tubes marked as „Sample‟. 4ml of biuret reagent was added to each of the test-tubes marked as „Blank‟, „Standard‟ and „Sample‟. The absorbance was read by UV Spectrophotometer at wavelength of 550nm. The

Protein content can be calculated by following calculation.

$$\text{Protein content} = \frac{\text{O.D. of Test}}{\text{O.D. of Standard}} \times \text{Conc. of Standard} \times \text{Dilution Factor}$$

Estimation of Phenol Content Using UV Spectrophotometer

Phenol is used as a preservative in the polysaccharides vaccines and antisera. Estimation of phenol content test performed on the Typhoid vaccine by using UV spectrophotometer (Oostra, GM, *et. al*, 1978). Phenol present in the vaccine sample combines with 4-aminoantipyrine which forms a complex. Potassium ferrocyanide acts as an indicator and gives color to the complex as an end point (*Skoog, DA, et al.*, 2007). Switch on the UV spectrophotometer 15 minutes prior to performing the test. 2.9ml of borate buffer and 0.1ml of D.W. were added in test-tube marked as „Blank". 2.9ml of Borate buffer and 0.1ml of Standard solution (0.25%) were added in the test tube marked as „Standard". 1:10 dilution were prepared of vaccine samples and added 2.9ml of buffer and 0.1 ml each of the diluted samples in the test-tube marked as; Sample". 3ml of 4-aminoantipyrine (0.1%) and 3ml of potassium ferrocyanide (1%) were added to each of the test-tubes marked as „Blank", „Standard" and „Sample". The absorbance was read in UV spectrophotometer at wavelength of 550nm. The phenol content can be calculated by following calculation.

$$\text{Phenol Content} = \frac{\text{O.D. of Test}}{\text{O.D. of Standard}} \times \text{Conc. of Standard}$$

Estimation of Molecular Size Distribution of Meningococcal-A Vaccines Using HPLC

Meningococcal vaccines containing highly purified high molecular weight capsular polysaccharides of sero groups A, C, Y and W 135 are commercially available. They are supplied as lyophilized monovalent A, monovalent C, divalent AC or tetravalent A-C-Y-W135 products (Wiechelman, KJ, *et al.*, 1988).

Preparation of mobile phase

Dissolve 12.70gm of disodium hydrogen phosphate, 3.2gm of sodium dihydrogen phosphate and 88.40gm of sodium chloride in 900ml of MQW. Adjust the pH to 7.2 with 1N NaOH. Make up the final volume to 1000 ml with MQW (Lloyd, RS and Dolan, JW, 2007).

Method

Switch on the HPLC instrument was switched on and check all the connections. The test samples were diluted (Acetones and Dextran) with MQW to 100µl. Prepare the mobile phase was prepared in MQW and filtered it in the solvent filtration unit. The blank (Mobile Phase), vaccine sample, dextran and acetone (100µl each) were transferred in HPLC vials and placed in the injection chamber and run. Connect the column was connected. The pump was purged and primed with water and mobile phase. The procedure was repeated until there were no air bubbles inside the tubing. The channels were selected on the system after the completion of the run. Channels corresponding to the acquired RI data and extract the chromatogram were selected and double click the row. The elution

volume (Ve) was measured at the main peak of polysaccharide elution curve and the Distribution Coefficient (K_D) was calculated by using the following equation.

$$K_D = \frac{Ve - Vo}{Vt - Vo}$$

Where

Ve - Elution volume

Vt - Total bed volume

Vo - Void volume

K_D - Distribution coefficient

In-vitro Potency of Hepatitis B Vaccines Using AxSYM System

Hepatitis B envelope protein or surface antigen (HBsAg) is composed of three related envelope proteins covalently linked together. Validated AxSYM system used for estimation of in-vitro potency assay (Izquierdo, M and Garcia, Y, 2001). In this method dilutions of the sample were prepared in the ratio of 1/1000, 1/2000, 1/4000, 1/8000 using PBS+2%BSA solution and similar dilutions were prepared for reference standard. The sample was put in the sample cups as per order and reagent pack in reagent vessel. The ordered in which sample were being put in the sample cups entered. The process by Run button was stared. The final results obtained were observed.

RESULTS

The qualitative and quantitative analytical methods performed in this study which was showed the best result and the maximum presence of constituent of vaccines. The free formalin content value in DTP vaccine was 2.0µg/ml which was confirmed by the qualitative method (Table 1). The thiomersal content in DTP, protein content in rabies and phenol content value in typhoid vaccine were confirmed by the quantitative method using UV spectrophotometer. The findings were obtained 0.0107%, 0.657% and 0.230 respectively (Table 2).The molecular size distribution of meningococcal-vaccines was 97.50% before the $0.5K_D$ which was confirmed by the qualitative method using HPLC. The HPLC chromatograms of dextran, acetone and meningococcal-A background solution were shows the sharp peaks (Figure 1, 2, 3 respectively). *In-vitro* potency of Hepatitis B vaccine can be confirmed by the AxSYM system. The finding was obtained relative potency 0.9769 %, estimated potency 19.54mcg/ml. The Lower Confidence Limit (95%) = 0.951(19.030mcg/ml), % Relative Potency = 97.40 and Upper Confidence Limit (95%) = 1.003 (20.060 mcg/ml), % Relative Potency = 102.67 (Table 3).

DISCUSSION

The qualitative and quantitative analysis of the vaccines shows the best result in terms of linearity, accuracy, precision and quality rather than the previous methods. Vaccines contained thiomersal, protein and phenol are estimated by different method i.e. visual method, simple spectrophotometer and derivative UV-spectrophotometer. But UV-spectrophotometer method of analysis is more economic and simpler. Under computer controlled instrumentation it play very important role in the analysis of vaccine samples. The results were within the acceptable range i.e. < 200µg/ml, 0.005%- 0.02%, >17% and > 0.25% for free formalin, thiomersal, protein and phenol content.

Meningococcal vaccine was analyzed by high performance liquid chromatography for the determination of size distribution to the polysaccharides. When separation of target compound from impurities in sample matrix is needed, HPLC offer better separation and therefore better sensitivities (Baskaran, S and Bolan, NS, 1998). The results showed sharp peak, have high resolution power, superior refractive index detector and rapid monitoring techniques than the other method used for the vaccine like gel filtration and ion exchange chromatography.

AxSYM system is the important techniques for the determination of potency of the vaccine rather than the *in-vivo* (animal study) method. The *in-vivo* method is a time-consuming and labor intensive process and the animal ethics committee makes the rule to avoiding the animal death. When the *in-vitro* study has done its very pain full to the animals due to scarify the animals and the variations occur in the results. But the results obtained of the hepatitis B vaccine by the *in-vitro* method which is more accurate and precious than the *in-vivo* study. Formalin is analysis by the qualitative method. The results obtained of the Diphtheria Tetanus and Pertussis vaccine for free formalin is more accurate and well with in the range rather than the pervious simple visual method. In this project study it was confirmed that these qualitative and quantitative analysis of various constituents of vaccines by using analytical techniques shows the best results than the visual inspection and old method which are used to estimation the constituents of vaccines. The result was within the acceptable range of less than 1%. In the present study suggested that all the findings were obtained after quantitative and qualitative analyzing the constituent of vaccine well with in acceptable range according to the IP, 2007.

CONCLUSION

In the present study it may be concluded that the analytical techniques latest model i.e. Ultra-Violet spectrophotometer, High Performance Liquid Chromatography and AxSYM system were used to analysis the chemical constituents of vaccines which are more economical, simpler and show the best result in terms of linearity, accuracy, precision and quality for the vaccines rather than the other previous methods such that visual inspection, colorimetric, simple spectrophotometer, gel filtration and animal testing.

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Table 1: Estimation of free formalin content

S.No.	Product	Free formalin content (µg/ml)	Mean(µg/ml)
1.	DTP	2.0	2.0
		2.0	
		2.0	

Table 2: Estimation of thiomersal, protein and phenol content

S.No.	Product	Estimation content	O.D. of Standard	Mean value (%)
1.	DTP	Thiomersal	0.377	0.0107
2.	Rabies	Protein	0.2804	0.657
3.	Typhoid	Phenol	0.319	0.230

Table 3: *In vitro* potency assay of HBsAg vaccine (rDNA)

S.No.	Product	Dilution	Potency			
1.	Reference Standard	1:1000	10.130	9.070	9.770	9.180
		1:2000	15.690	15.510	15.870	14.130
		1:4000	22.69	23.32	23.12	22.24
		1:8000	68.18	67.87	67.18	66.770
2.	Hepatitis B	1:1000	9.070	9.690	9.890	9.600
		1:2000	14.730	14.860	15.120	14.550
		1:4000	23.46	22.77	21.39	22.6
		1:8000	63.56	65.38	65.71	68.690

Molecular size distribution of meningococcal-vaccines:

2 DEXTRAN			
Sample Name:	**Dextran**	*Injection Volume:*	**100.0**
Vial Number:	**2**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**280**
Control Program:	**MENINGOCOCCAL_1**	*Bandwidth:*	**4**
Quantif. Method:	**MEN_ Intg**	*Dilution Factor:*	**1.0000**
Recording Time:	**10/10/2010 13:17**	*Sample Weight:*	**1.0000**
Run Time (min):	**60.00**	*Sample Amount:*	**1.0000**

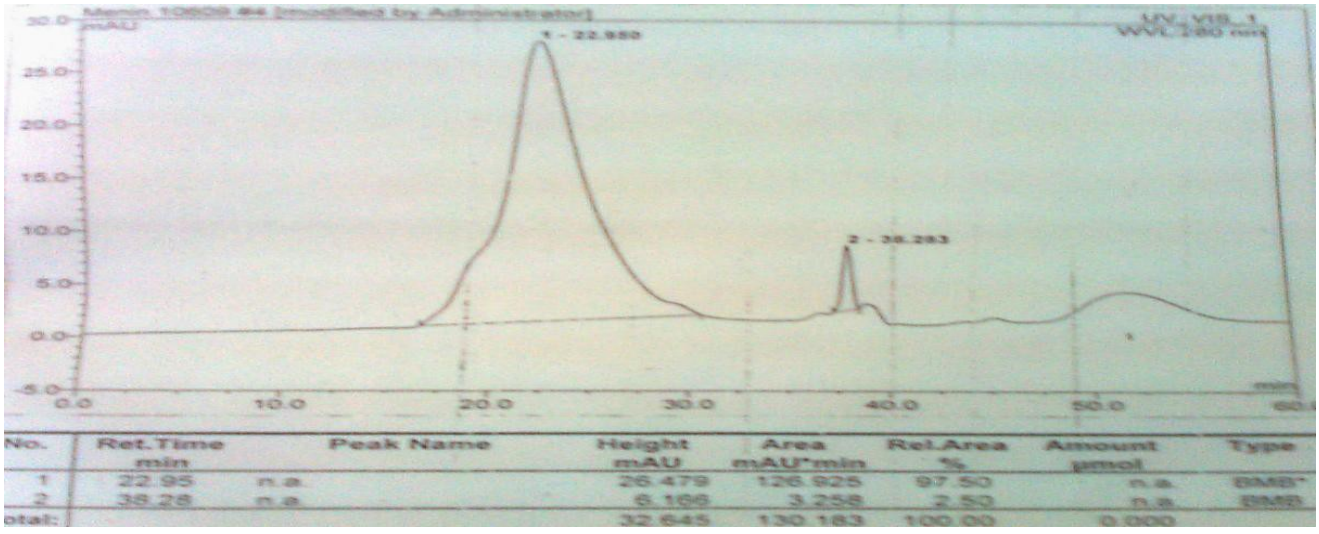
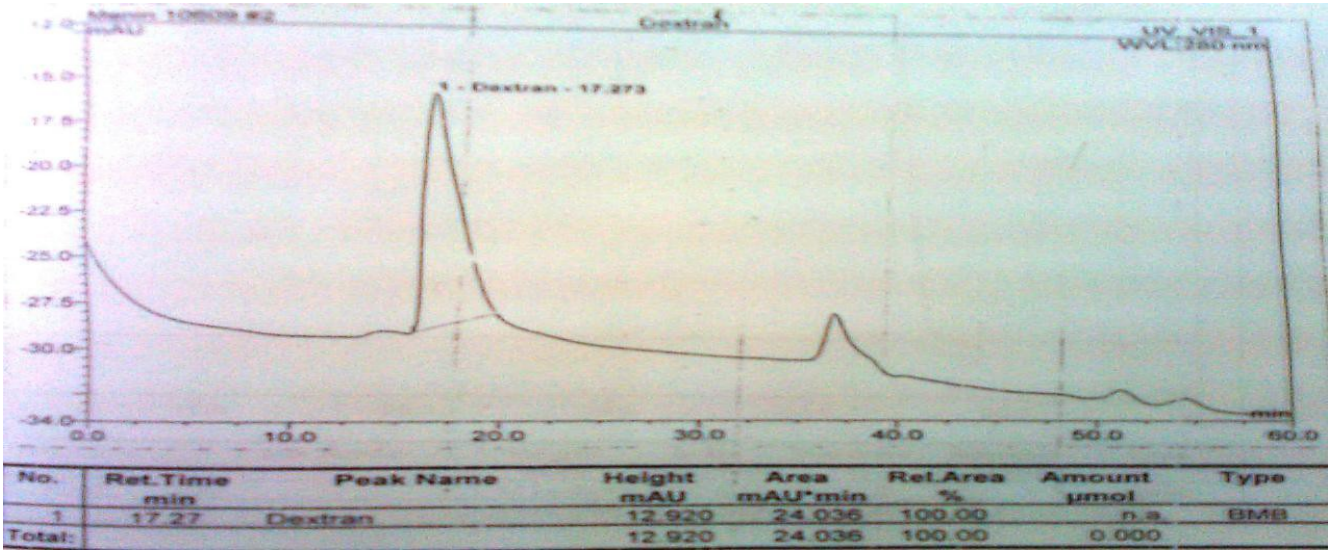


Figure1: The HPLC chromatograms of (Dextran) background solution

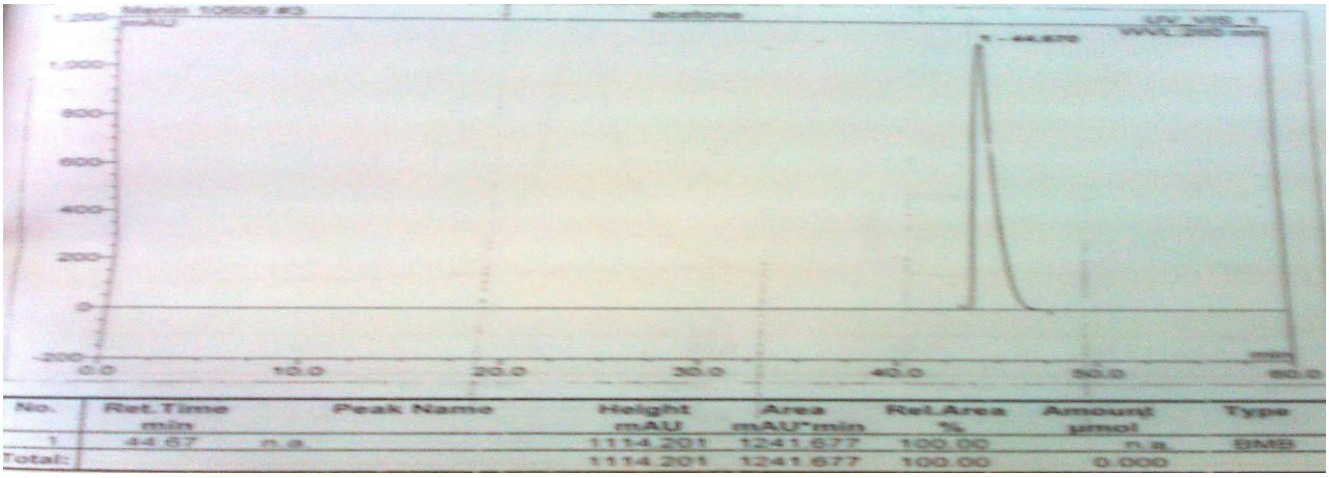
3 ACETONE			
Sample Name:	**Acetone**	*Injection Volume:*	**100.0**
Vial Number:	**3**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**280**
Control Program:	**MENINGOCOCCAL_1**	*Bandwidth:*	**4**
Quantif. Method:	**MEN_ Intg**	*Dilution Factor:*	**1.0000**
Recording Time:	**10/10/2010 14:24**	*Sample Weight:*	**1.0000**
Run Time (min):	**60.00**	*Sample Amount*:	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount µmol	Type
1	17.27	Dextran	12.920	24.036	100.00	n.a.	BMB
Total:			12.920	24.036	100.00	0.000	

Figure2: The HPLC chromatograms of (Acetone) background solution

4 Men A Samples 2			
Sample Name:	**Men A Samples 2**	*Injection Volume:*	**100.0**
Vial Number:	**4**	*Channel:*	**UV_VIS_1**
Sample Type:	**Unknown**	*Wavelength:*	**280**
Control Program:	**MENINGOCOCCAL_1**	*Bandwidth:*	**4**
Quantif. Method:	**MEN_ Intg**	*Dilution Factor:*	**1.0000**
Recording Time:	***10/10/2010***	*Sample Weight:*	**1.0000**
Run Time:	***60.00***	*Sample Amount*:	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount µmol	Type
1	44.67	n.a.	1114.201	1241.677	100.00	n.a.	BMB
Total:			1114.201	1241.677	100.00	0.000	

Figure3: The HPLC chromatograms of (Men A sample 2) background solution

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