Volume 1 Issue 1

BIOCHEMISTRY
&
MOLECULAR BIOLOGY *Letters*

Full Paper

BCAMBL, 1(1), 2015 [050-056]

Organic solvents determination in CNBr Sepharose CL-4B and immunoaffinity matrix

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ABSTRACT

In general, the effects of organic solvents exposure are narcosis, anesthesia, central nervous system depression, respiratory arrest, unconsciousness, and death. Many countries have issued several regulations to control and reduce the exposition of the personnel to this kind of solvents. Matrix and researchers are exposed to organic solvents during the immunoaffinity chromatography gel manufacture. In this case the manufacture of the immunosorbent CB.Hep-1 used for the immunopurification of the HBsAg, active raw material of Cuban vaccines against Hepatitis B. In this process any organic solvents are used. Gas chromatography and total organic carbon assay were used to determine the presence of Ethanol, Acetic acid, Acetonitrile and Acetone. CNBr Sepharose CL-4B showed an average 274.2 ppm of Ethanol; 28 ppm Acetic acid; 288 ppm Acetone/ Acetonitrile. In addition to this very low level of organic solvents in activated matrix, the presence of them were not detected in the immunosorbent therefore the risk to organic solvent exposure in the final purified antigen does not exist.

KEYWORDS

Organic solvents;
Gas chromatography;
TOC;
Immunosorbent CB.Hep-1;
Immunopurification.

INTRODUCTION

Know about the presence of residues organic solvents is a very important aspect in the pharmaceutical productions. It is even more important if the product is involved in the manufacturing of a vaccine for healthy people; which should be submitted to numerous regulations and rigorous controls[1].

The term "organic solvent" refers to a group of volatile compounds or mixtures that are relatively stable chemically and that exist in the liquid state at temperatures of approximately 0° to 250°C (32° to 482°F). Usually organic solvents are classified as aliphatic, cyclic, aromatic, and halogenated hydrocarbons, ketones, amines, esters, alcohols, aldehides, and ethers, but also many common organic solvents often exist as mixtures or blends of chemical compounds[2].

The acute neurotoxic effects of organic solvent

exposure in workers and laboratory animals are narcosis, anesthesia, central nervous system (CNS) depression, respiratory arrest, unconsciousness, and death. Chronic exposure animal studies with a limited number of organic solvents support also the evidence for peripheral neuropathy and mild toxic Encephalopathy in solvent-exposed workersp[3].

Epidemiologic studies of various groups of solvent-exposed workers have demonstrated statistically significant chronic changes in peripheral nerve function (sensory and motor nerve conduction velocities and electromyography abnormalities) that persisted for months to years following cessation of exposure. These studies have also shown significant increase in neurobehavioral effects in workers chronically exposed to organic solvents. These effects include disorders characterized by reversible subjective symptoms (fatigability, irritability, and memory impairment), sustained changes in personality or mood (emotional instability and diminished impulse control and motivation), and impaired intellectual function (decreased concentration ability, memory, and learning ability)[4].

The affinity chromatography is a technique with a higher resolution power, for purification of proteins and other biomolecules. This technique is based on interactions between two active biologically molecules, which is characterized by an extraordinary selectivity. Examples of this type of interaction are the enzymatic reaction, controlled by the affinity among enzyme and substrate or to one inhibitor, or one cofactor, an also an antigen by its antibody. The matrix used in affinity chromatography can be synthetic or of natural origin. Inside of supports of natural origin the more used are polisacaridics, the based on agarose, the cellulose, and the dextrin. On the other hand poliacrilamide, and esferones are also frequently used[5]. *The activation of all of them needs organic solvents*.

In the manufacture process of the immunosorbent CB.Hep-1 for the immunopurification of the HBsAg, Sepharose CL-4B is employed, which contains Ethanol as preserve and others organic solvents are also used during the activation of the support to immobilise the antibodies.

The objective of this work was demonstrated that the presence of these solvents in the activated matrix and also in the immunogel is under the danger limit for the workers for the healthy people who will receive the vaccine.

MATERIALS AND METHODS

Monoclonal antibody

CB.Hep-1 Mab was previously generated by Fontirrochi et al.[6]. It recognizes the "a" determinant of the HBsAg[7]. CB.Hep-1 was purified from ascitic fluid by Protein-A affinity chromatography. Finally, the Mab was filtered through a 0.2µm pore-sized membrane (Sartorius, Goettingen, Germany) and stored at 4ºC. The Mab was dialyzed in order to exchange the buffer 20 mM tris-150 mM NaCl pH 7.6 by coupling buffer 0.1 M Na_2CO_3-0.1 M $NaHCO_3$ -0.5 M NaCl pH 8.3 by mean of gel filtration chromatography. Pre-packed disposable columns PD-10 (Amersham-Pharmacia Biotech, Uppsala, Sweden), with 9.1 ml of swollen Sephadex G-25 M were used. The protein concentration (5 ±0.2 mg/ml) was determined according to the methods described by Lowry et al.[8]. This Mab is used as immunoligand in the downstream purification process of rec-HBsAg, employed for a commercially available recombinant Hepatitis B virus vaccine (Heberbiovac HBTM, Heber Biotec S.A., Cuba)[9].

Matrix activation

The Sepharose CL-4B (Amersham-Pharmacia, Upssala, Sweden) was moderately activated (7-8 µmoles/ml) with Cyanogen bromide (Merck, Darmstadt, Germany) using a modified procedure already described by Axen and Porath[10]. A modified Koenig reaction determination of cyanate ester concentration on the support was made[11].

Immunosorbents preparation

The CNBr activated Sepharose CL-4B matrix was wetted in 1 mM HCL for 15 minutes, and washings with 0.1 M Na_2CO_3-0.1 M $NaHCO_3$-0.5 M NaCL pH 8.3 were performed. The Mab was coupled by covalent bonds on the support at pH 8.3 during 2 hours at 25°C by gentle stirring. The coupling efficiency (δ) was determined by an indirect

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method, following the formula: φ (%) =δ/χx100. Where, δ is the amount of coupled protein determined as the difference between the original amount of ligand (χ) and the amount detected in the filtration and washings fractions after the coupling. Adding of 0.1 M glycine pH 8.0 blocked free reactive groups. Five alternate washings with 0.1 M $C_2H_3O_2Na$-0.5 M NaCL pH 4.0 and 0.1 M Na_2CO_3-0.1 M $NaHCO_3$-0.5 M NaCL pH 8.3 were made. Finally, the immunosorbents were washed and stored in phosphate buffered saline-0.01% Tiomersal pH 7.2 at 4ºC.

Gas chromatography

The samples were injected directly into a column Porapak N (2.1m of large x 4mm diameter intern) on the equipment Pye Unicam PU 4550 coupled Shimadzu C-R3A Chromatopac. The temperature of the column was 160 ^{0}C and the injector and detector 200 ^{0}C. The velocity of the nitrogen flow was 40 mL/min. The detectors the ionization of flame (FID) utilizes a velocity of air flow of 330 mL/min and a hydrogen velocity flow of 33 mL/min. The range of amplify was 10 and the attenuation of integrator was 6 for active matrix and 4 for immunosorbents. The flow of sample injected was always 10μL/min[12].

For organic solvents evaluation in the active matrix and immunosorbent samples, the water used in the wash was used as control. For washing the matrix and immunosorbents was used five volume of water/mL of gel.

Total organic carbon determination

The determination of total organic carbon (TOC) is performed by the wet oxidation method on an OI Analytical Model 1010 Total Organic Carbon Analyzer. The sample is injected into the instrument were it is acidified and purged of inorganic carbon. Sodium persulfate is added and this oxidant quickly reacts with organic carbon in the sample at 100 ^{0}C to form carbon dioxide. The carbon dioxide from the oxidized organic carbon is purged from the solution and detected by a no dispersive infrared (NDIR) detector that has been calibrated to directly display the mass of carbon dioxide detected. This mass is proportional to the mass of TOC in the sample.

RESULT AND DISCUSSION

Today the massive vaccination program is the more important instrument for the control in global scale of Hepatitis B. This strategy correctly apply can conduce to eradication of the infection by HBV how important casual agent of mobility, lost economic and mortality.[13]

The DNA recombinant technology and the application in the production of substances of therapeutic use how vaccines, insulin human, interferon, and other pharmaceutics products, make need development of procedure of purification. The process of chromatography of affinity in the purification, separation and assays of enzymes, hormones, proteins, antibodies and other molecules has to win significant interest in the industry biotechnology[14].

The method of CNBr matrix activation is quite popular for affinity chromatography. This is a simple procedure to immobilize first amine, in a short period of time (0.2-0.4 hours) with good efficiency of immobilization. Principal disadvantages are the great toxicity of the reactives and the ligand linkage[5].

On the base on the identified adverse effects to organic solvent exposure, the National Institute for Occupational Safety and Health (NIOSH) of U.S.A recommends that employers use engineering controls, personnel protective equipment and clothing, and worker education programs to reduce exposure to organic solvents[15].

Chronic neurotoxicity in workers exposed to organic solvents over a period from months to years includes (1) peripheral neuropathies such as axonal degeneration seen in workers exposed to hexacarbon solvents (e.g., n-hexane, methyl n-butyl ketone); (2) Type 1 CNS symptoms such as fatigability, irritability, and memory impairment, and (3) Type 2 mild toxic Encephalopathy, including sustained personality or mood changes such as emotional instability, diminished impulse control and motivation, and impairment in intellectual function manifested by diminished concentration, memory, and learning capacity[16].

Inhalation and percutaneous absorption are the primary routes of solvent uptake into the peripheral blood, which begins within minutes of the onset of

TABLE 1 : Gas chromatography retention times of organic solvent standards

Standard	Retention time (min)	Area under curve	Concentration (ppm)
Ethanol	5.2	2659491	500
Acetonitrile	7.3	2654899	500
Acetone	7.6	3189073	500
Acetic acid	24.5	4508993	1000

exposure[17]. Uptake by inhalation is the principal route and depends on the following: solvent concentration in inhaled air, blood/air partition coefficient of the solvent, and duration of exposure[18].

Metabolism usually results in the detoxication of the organic solvent through formation of water-soluble compounds that are excreted through urine or bile[19]. However, metabolism may also produce reactive intermediate metabolites that are more toxic than the parent compound. These metabolites are capable of covalently- binding to essential macromolecules (e.g., proteins, RNA, and DNA) and producing toxic effects'[17]. For example, n-hexane and methyl n-butyl ketone (solvents that produce peripheral neuropathies in exposed workers[14] both are metabolized to 2,5-hexanedione[20], which has shown to have a greater neurotoxic potency than either parent compound[3].

The Acetone, Acetonitrile, Ethanol, and Acetic acid may cause effects on the central nervous system, liver, kidneys, blood, bone marrow, and respiratory and gastrointestinal tracts. These substances may also cause effects on the cellular respiration (inhibition), resulting in impaired functions. Exposure at high levels may also provoke death according to the International Chemical Safety Cards ICSC: 0087.

The Acetone is not classified as a human carcinogen; the toxicological information in rat is oral way LD_{50} is 5800 mg/kg and inhales LC_{50} 50-100 mg/m^3[19]. Acetonitrile concentrations > 500 ppm may cause irritation of mucosal membranes and also can produce nausea, convulsion and death. It is toxic in human by ingestion or skin contact, the RFD 0.006 mg/kg based on diminution of red cells account, and hepatic lesion in rat[20].

This study started from two different samples, first one CNBr Sepharose CL-4B matrix. This Sepharose CL-4B normally contains Ethanol 20% as preserve and in its activation Acetonitrile, Acetic acid and Acetone (this in major concentration) are needed. The second one is the immunosorbent that requires the activated matrix after several washes with aqueous solutions before the coupling of the monoclonal antibody.

TABLE 1 and Figure 1 shows the chromatograms and retention times of the standard solvents measured in gas chromatography. These results showed a retention time of 5.2 and 24.5 minutes for Ethanol and Acetic acid respectively. However in case of the Acetone and the Acetonitrile the retention times were almost similar (7.3-7.6), reason why this method can not be used to discriminate among these tow solvents.[21]

TABLE 2 shows the amount of organic solvent residual in the CNBr Sepharose CL-4B. No more than 350 ppm of Ethanol was found in the washed of 5 different activated matrixes which is under the limit of exposure to this solvents, 1000 ppm in 8 hours[22]. In addition the Acetone and Acetonitrile level was under 440 ppm. Both organic solvents have the same retention time in gas chromatography (TABLE 1) but the great part of this fraction should correspond with the Acetone due to it is used in a major proportion during the process (24L of Acetone/1L Acetonitrile). Nevertheless the process is safe because the limit of exposure for Acetone is 750-1000 ppm in 8 hours and 40 ppm for Acetonitrile. In three samples the Acetic acid was not detecTABLE while in the others the quantities were 22 and 34 ppm. In principle this level could be dangerous because the limit of exposure according to United State Department of Health and Human Service is 10 ppm but the Oral LD_{50} in rat is 3310 mg/Kg and the inhalation LD_{50} in mouse is 5620 ppm/h[23]. Reason why these values of Acetic acid are not dangerous at all considering that the time of exposure is short and all operation are performed under powerful gas extraction cham-

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TABLE 2 : Organic solvent residues present in to CNBr Sepharose CL-4B

Samples	Retention time (min)	Area under curve	Concentration (ppm)
Water	ND	ND	ND
	ND	ND	ND
	ND	ND	ND
CNBr Sepharose CL-4B (1)	5.3	908551	171
	7.7	1267196	217
	24.9	100745	22
CNBr Sepharose CL-4B (2)	5.2	1547776	291
	7.7	1662708	285
	24.8	155502	34
CNBr Sepharose CL-4B (3)	5.2	1599803	301
	7.7	2150416	368
	25.0**	ND	ND
CNBr Sepharose CL-4B (4)	5.3	1744988	328
	7.7	1468417	251
	ND	ND	ND
CNBr Sepharose CL-4B (5)	5.2	1490884	280
	7.7	1866734	319
	ND	ND	ND

ND: not detectable; (**) To small picks, this could not be quantified

TABLE 3 : Organic solvents residues in the immunosorbents CB.Hep-1 measured by gas chromatography

Samples	Retention time (min)	Area under curve	Concentration (ppm)
Water	ND	ND	ND
	ND	ND	ND
	ND	ND	ND
Immunosorbent CB.Hep-1 (1)	ND	ND	ND
	ND	ND	ND
	ND	ND	ND
Immunosorbent CB.Hep-1 (2)	ND	ND	ND
	ND	ND	ND
	ND	ND	ND
Immunosorbent CB.Hep-1 (3)	ND	ND	ND
	ND	ND	ND
	ND	ND	ND
Immunosorbent CB.Hep-1 (4)	ND	ND	ND
	ND	ND	ND
	5.2	120619	4
Immunosorbent CB.Hep-1 (5)	ND	ND	ND
	ND	ND	ND

ND: not detectable

bers.

As it shown in TABLE 3 and Figure 1 not organic solvent was detected in the immunosorbents. Only in one case the Ethanol was found in a very low concentration (4 ppm). These results confirm that the immunosorbent is quite safe in terms of solvent organic presence. Therefore it does not represent a danger at all for the production of a vaccine for human use. Additionally the results measured by gas chromatography were corroborated by the total organic carbon method, which is an indirect measure of organic solvents because it is not possible to determine which organic solvent is present in the sample. Figure 2 shows that the majority of organic carbon is in the samples of the CNBr Sepharose CL-4B (10000 ppb) while in the immunosorbents the organic carbon was very low in all samples evaluated (<500 ppb). Therefore these results also confirm that there is not presence of organic solvent in the final preparation of the immunogels.

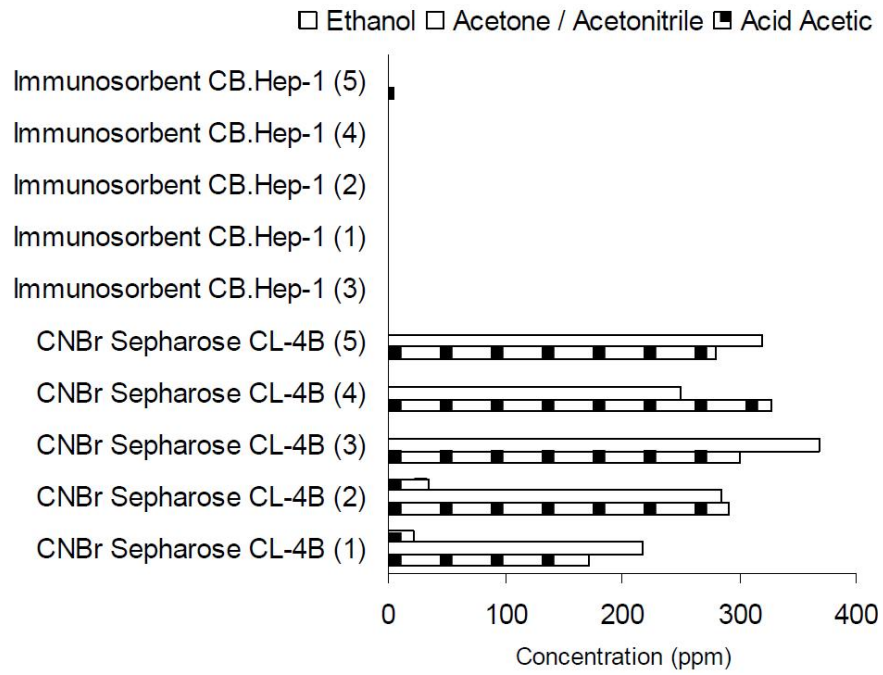

Figure 1 : Presence of organic solvents in samples of CNBr Sepharose CL-4B and Immunosorbents CB.Hep-1

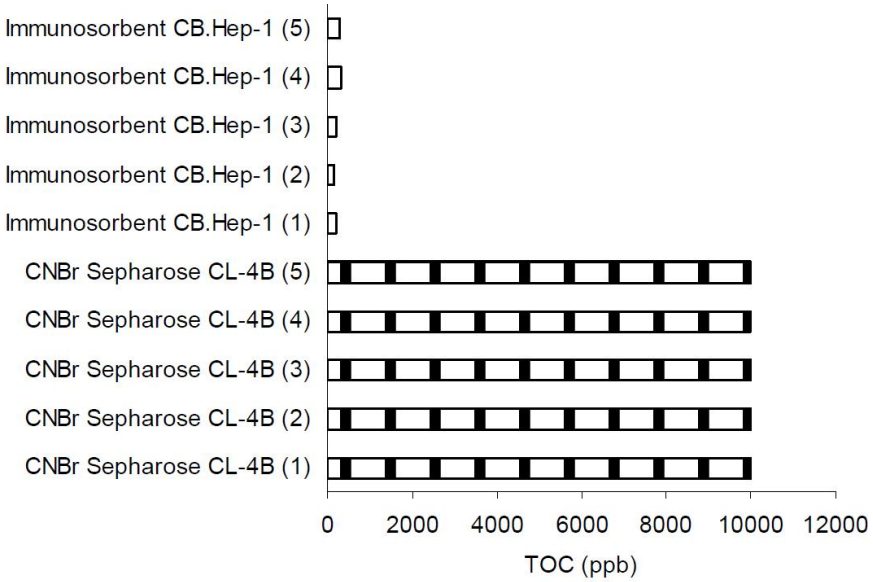

Figure 2 : Total organic carbon in CNBr sepharose CL-4B and immunosorbents CB.Hep-1

ABBREVIATIONS

NIOSH (U. S Department of Health and Human Services). OSHA (U.S Department of labor and is responsible for creating and enforcing workplace safety and health regulations.

ppm (part by million).ppb (part by billion). HBsAg (Hepatitis B surface antigen).

Mab (Monoclonal antibody).

TOC (Total Organic Carbon).

CONCLUSIONS

In this work was demonstrated that the level of organic solvents in the activated matrix and also in the immunogel use for immunopurification of HBsAg grow material active of Cuban vaccine against Hepatitis B is under the danger limit for the workers and for the healthy people who will receive the vaccine.

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