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DETERMINATION OF NITROGEN MUSTARD BY USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

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NITROJEN MUSTARDIN YÜKSEK BASINÇLI SIVI KROMATOGRAFİSİ VE KÜTLE SPEKTROMETRİSİ KULLANILARAK ÖLÇÜMÜ

Özet: *Nitrojen Mustard (Mekloretamin; bis[2-kloroetil] metilamin; HN2) kanser tedavisinde bir kemoterapotik ajan olarak kullanıldığı gibi, aynı kükürtlü mustardın bir azot analogu şeklinde kimyasal bir silah olarak da kullanılmaktadır. Bu çalışma derivatize edilmiş HN2'nin plazmadan ekstraksiyonu ve Yüksek Basınçlı Sıvı Kromatografisi (HPLC) ve Kütle Spektrometrisi (MS) kullanılarak ölçümü esasına dayanmaktadır. Bu yöntemde, plazma örneklerinde HN2'nin ekstraksiyonu için minikolonlar kullanıldı ve derivatizasyon işlemi için Dietil-ditiyokarbamik acid (DDTC) ilave edildi. Bu işlemde, DDTC molekülü, HN2'nin her bir dalındaki klor atomu ile yer değiştirerek stabil HN2-DDTC2 adduktları oluşturmaktadır. Oluşan bu maddeyi, dolayısıyla HN2'yi ölçmek için gradiyent elüsyon yöntemiyle HPLC tekniği kullanılmıştır. Bu teknikle, bu maddenin tutunma zamanı 15.2 dak ± 1.0% bulunurken, bu molekül MS kullanılarak konfirme edilmiştir. Elde edilen verilere göre, gün içi CV %3.6-%4.9 arasında, günlerarası CV ise %5.1-%6.8 arasında bulunmuştur. Ayrıca, HN2'nin saptanma limiti 10.0 µg/ml, doğrusallık aralığı ise 10-100 µg/ml olarak bulunmuştur. Sonuç olarak; DDTC ile derivatize edilme durumunda HN2'nin ölçümünde ve oluşan HN2-DDTC2 adduktlarının saptanmasında HPLC tekniğinin, bunun doğrulanması amacıyla da MS'in kullanılabileceği sonucuna varılmıştır.*

Anahtar Kelimeler: *Nitrojen Mustard; HPLC; Kütle Spektrometrisi; DDTC; derivatization.*

Summary: *Nitrogen Mustard (Mechlorethamine; bis[2-chloroethyl] methylamine; HN2) is both a chemotherapeutic agent used in control of cancer and also the chemical weapon as being a nitrogen analog of sulphur mustard. This study is based on extraction of derivatized HN2 from plasma and its determination by High Performance Liquid Chromatography (HPLC) and Mass Spectrometry (MS). In this study, minicolumns were used for extraction of HN2 in plasma samples and Diethyl-dithiocarbamic acid (DDTC) was applied for derivatization. At this derivatization process, one molecule of DDTC is being reacted with each arm of HN2, displacing a chlorine atom to form a stable disubstituted adduct (HN2-DDTC2). In order to determine this adduct and to detect the HN2 indirectly, we used HPLC technique including gradient elution. By HPLC, the retention time of this adduct was found as 15.2 min ± 1.0% within day and confirmed by using mass spectrometry which enabled our method superior compared to the other studies. According to data obtained, the intraassay CV was between 3.6 and 4.9 % and interassay CV was from 5.1 to 6.8 %. In addition, we determined the detection limit of HN2 as 10.0 µg/ml and the linearity range between 10-100 µg/ml. As a result, HPLC method can be used for determination of HN2 on condition that HN2 would be derivatized with DDTC and the available HN2-DDTC2 could be detected and confirmed by using mass spectrometry.*

Key Words: *Nitrogen Mustard; HPLC; Mass Spectrometry; DDTC; derivatization*

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INTRODUCTION

Nitrogen mustard, mechlorethamine [(bis-2-chloroethyl) methylamine; HN2] is a reactive alkylating agent which has been used in cancer chemotherapy for over 50 years (1). It has been suggested that HN2 acts via covalent modification of DNA. Early studies on the reaction of HN2 with DNA and RNA provided evidence that monoalkylation product and cross-link were formed at N-7 of guanine (2,3,4). Nitrogen mustards are themselves highly carcinogenic apart from becoming bifunctional alkylating agents (5). Due to high chemical reactivity and presumably having short half-life in human, HN2 exhibits limited therapeutic activity against the major solid tumours. Therapeutically, effective antitumour activity of HN2 suggests that it has two functional groups involved in the formation of crosslinks between the macromolecular sites (6,7). In this study, we aimed to find sensitive HPLC method, along with a reproducible and efficient sample preparation technique for the determination of HN2 in plasma.

MATERIALS AND METHOD

a. Materials: Diethyldithiocarbamic acid-Sodium salt (DDTC) and Nitrogen Mustard (Mechlorethamine hydrochloride salt) were from SIGMA Chemical Co. Methanol, acetonitrile and orthophosphoric acid (HPLC reagent grade) were from MERCK Co. (Darmstadt, Germany). Water was deionized and bidistilled in a quartz glass. All other chemicals were of the highest grade available commercially.

b. Precautionary Steps And Processes: Nitrogen mustard are hazardous substances and carcinogen materials (8). So, while preparing reagent containing HN2, compound was weighed out in a safety cabinet and transferred directly into a volumetric flask. Dilutions were performed as quickly as possible with small amount of solutions to avoid time delay and chemical decomposition. Contaminated materials were treated with the solution of NaOH (5M) / Ethanol (1:10; v/v) (9, 10). During working throughout, full protective mask and gloves were worn for protecting from the harmful effects of HN2 (11,12).

c. Derivatization Procedure: 100 µl DDTC freshly prepared in a 0.1 N Natrium hydroxide (100 mg/ml) was added to 1 ml of plasma containing nitrogen mustard at different concentrations from 1 to 100 µg. Specimens containing HN_2 were then incubated for 30 min at 37 ^{0}C (Figure 1).

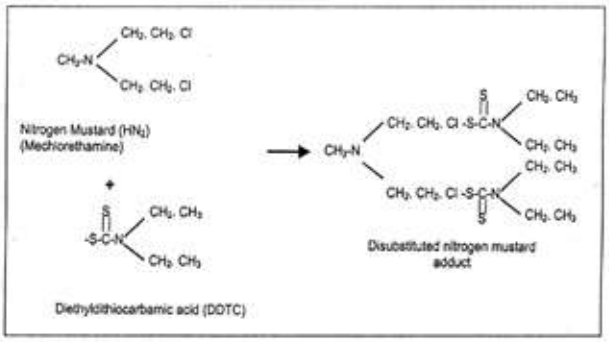

Figure 1. Proposed reaction mechanism of DDTC with HN_2

d. Sample Preparation: After derivatization, DDTC adducts of HN_2 were extracted from plasma samples by using "Bond Elut" minicolumns (SUPELCO^R) . Minicolumns were at first activated with 2 ml of water prior to loading 1 ml of sample. The columns were then washed with 5 ml of water and kept at room temperature at dark. Finally, the derivatives were eluted with 2 ml of acetonitrile which was evaporated to dryness in a centrifugal vacuum evaporator (Speed Vac-Plu-SC 110 A) joined Refrigerated Vapor Trap (RVI 4104) at 40 ^{0}C. Residues were dissolved in 200 µl of methanol.

e. High-Performance Liquid Chromatography: We used HPLC system consisted of a VARIAN 5000 Liquid Chromatography equipped with a Rheodyne 7125 sample injector (20 µl loop) connected to an optical detector (UV-VIS 200; LINEAR Corp. Reno, NV.USA). The seperation column used here was (4 µm Supersphere 100, RP-18, 250x4 µm I.D., Merck,

Darmstadt, Germany) with a guard column packed with the same matrix. The chromatograms were recorded by using a PC-based data acquisition and processing system (Chroma Set; Bar Spec; Israel).

Chromatography was applied by using four different mobile phase program including UV detector, seperately for each mobile phase application. Flow rates for all of them were 1 mL/min. Our chromatographic applications for determining HN2 were as follows:

- First application:The mobile phase consisted of a 50 mM KH2PO4 and acetonitrile mixture (55:45, v/v).

- Second application:The mobile phase included water-acetonitrile-acetic acid mixture.

- Third application: The mobile phase consisted of water-methanol-acetic acid mixture.

- Last application: Here, chromatography was carried out by a mobile phase using a gradient including 5 mM ortophosphoric acid (pH 3.0-Mobile phase A) and acetonitrile (Mobile phase B). The starting proportion of the gradient at zero time was 30 % B and 70% A. Mobile phase B was maintained constant for 4 min and then increased linearly to 100% B by 13 min, returning back to 30 %B by 20 min. A total run time for each assay was for 30 min.

Mobile phase components were degassed by making them vacuum prior to use. After full preparations, samples were injected to HPLC as 20 µl.

e. Mass Spectrometry: Mass spectral analyses were carried out by LKB mass spectrometer 2091 and scanned from 50-400 m/z using Electron Impact (EI) for ionization. Other operating conditions were as follows: Emission current, 1 mA; electron energy, 70 eV; Ion source temperature, 250 0C and Ion acceleration voltage was initially 3 kV .

RESULTS AND DISCUSSION

In this study, our aim was to establish an effective method by utilizing HPLC instrument for detecting or determining HN_2 being available in plasma. We had three targets for reaching the success. One of them was to separate HN_2 present in the biological materials with the highest efficiency. So, we used minicolumns having containing silica gel 2.4 ml capacity. The samples containing HN_2 at the different concentrations after derivatization were extracted from these columns. It has been suggested that the solid phase method using these columns did not extract any endogen component from plasma that could interfere with the identification of HN_2 (1,13). So, this would give permission plasma samples to be analyzed at the highest sensivity.

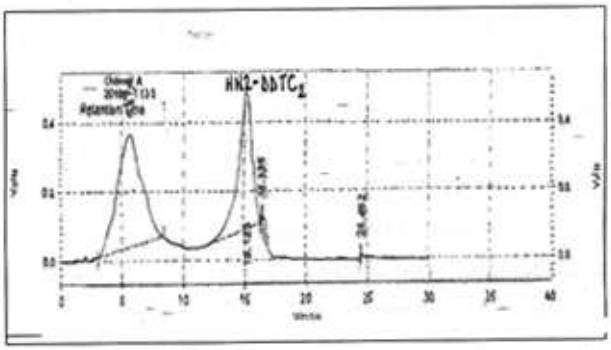

Figure 2. Chromatogram of nitrojen mustard (HN_2) after derivatization with diethyldithiocarbamic ecid in 0.1 N NaOH. Disubstituted nitrojen mustard has been determined to have 15.123 min. o retention ime

Our second target was to prevent the disappearance of HN2 from plasma due to either hydrolysis or covalent binding prior to HPLC analysis. For this reason, we used DDTC for derivatization of HN_2. Our aim here was to form disubstituted derivatives of HN2 which had UV spectrum at 276 nm in wavelength (Figure 2 and 3). In order to constitute disubstituted nitrogen mustard adduct, 100 µl DDTC freshly prepared in 0.1 N NaOH was added to 1 ml of plasma samples containing nitrogen mustard. For derivatization, specimens were incubated for 30 min at 37 ^{0}C. In this condition, to determine whether disubstituted nitrogen mustard adduct (HN2 –$DDTC_2$) have taken place or not, we analyzed these samples by using

mass spectrometry in terms of moleculer ion contents. Our results about the spectrum of mass spectrometry indicated that the end product of DDTC derivatization yielded a moleculer ion-MH+ at m/z 386 approximately. Therefore, disubstituted nitrogen mustard adduct was realized at this incubation conditions applied.

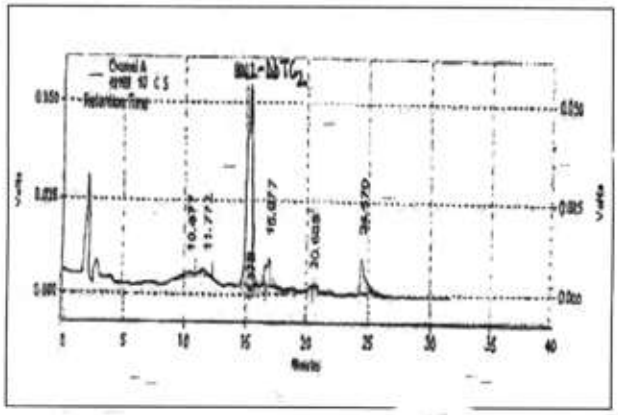

Figure 3. Chromatogram of disubstituted nitrogen mustard adduct (HN2-DDTC2) taking place after derivatization procedure between nitrogen mustard (HN2) and with diethyldithiocarbamic acid (DDTC2). Required data of chromatogram is being shown above figure.

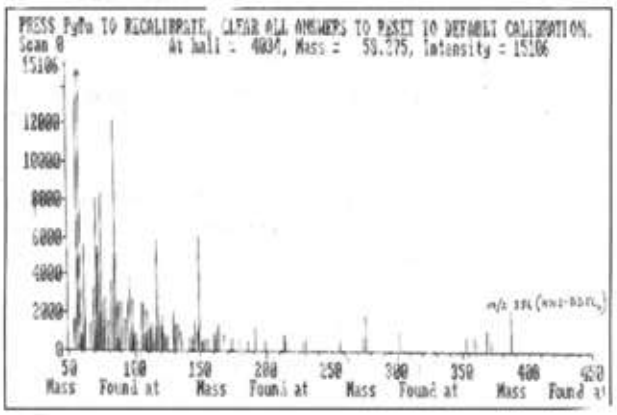

Figure 4. Structural identification of nitrogen mustard-DDTC2 derivatives has been evaluated by mass spectrometry, and molecular ions and electron impact mass spectra of the derivatives are illustrated in figure above.

Third target was to determine the best HPLC application program which would provide the smallest detection limit. For this aim, we applied different programs including different mobile phases and their applications. Among them, we approved the best detection program of HN_2 in HPLC employing gradient elution at a flow rate of 1 ml/min. In this program, 5 mM orthophosphoric acid and acetonitrile were used as mobile phase components. The derivatized HN_2 samples had a retention time (t_R) being present 15 min, but in order to confirm whether the retention time observed at the 15 min would be related to HN_2-$DDTC_2$, we collected the elution material taking place at the 15 min retention time and analyzed this elution sample by mass spectrometry in terms of moleculer ions (Figure 4). At the end of our evaluation, we arrived the decision that the peaks observed at the 15 min were concerning the HN_2- $DDTC_2$.

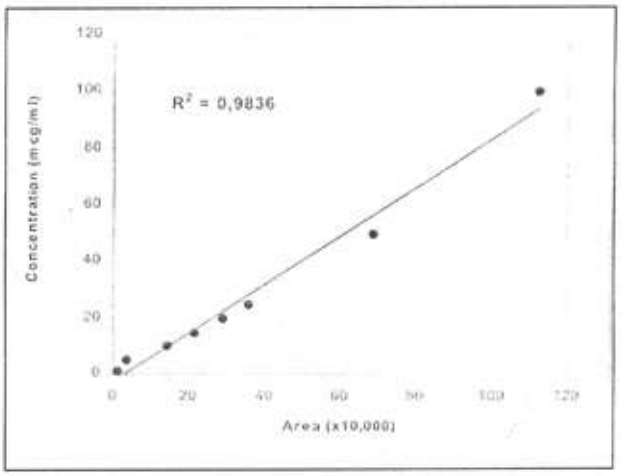

Figure 5. The calibration curve of HPLC method.

In order to evaluate the intra-day precision (expressed as CV) of HPLC method, we determined the values of the HN_2 at three different concentrations on four different days. Calibration results from 1 to 100 μg/ml, precision and recovery results were shown in Table 1 and Figure 5. Moreover, inter-day precision was evaluated determining three specimens of the HN_2 at the same concentrations within the day and was found between 3.6 and 4.9 %. For inter-day precision, the CV obtained was ranged from 5.1 to 6.8 %. On the other hand, the detection limit of the HN_2 was up to 10.0 μg/ml and linearity range was between 10 to 100 μg/ml on the conditions that we used for the HPLC method explained above. With respect to literature

sources regarding this issue, detection limit for HN_2 has been determined at different values ranged from 1 ng/ml to 25 μg/ml (1,3,4). The detection limit determined by us for derivatized HN2 was 10 μg/ml, and with regard to this value, we evaluated this method providing HN_2 to be analyzed with high sensitivity. Based on our observations and literature resources (1,13,14,15,16), we concluded that DDTC was the useful reagent for derivazation of HN2 prior to introducing to HPLC and minicolumn extraction method was available in terms of extraction efficiency and recovery.

***Table 1**: Intra-day and inter-day precision and recovery results of the modified and optimized HPLC methods.*

Concentration (μg/ml)	Recovery (%)	Precision (%)	
		intra-day	inter-day
1	0		
5	57.0 ± 12.5	3.6 ± 0.6	5.1 ± 1.6
10	82.0 ± 4.5	4.7 ± 1.2	6.2 ± 1.5
25	89.4 ± 3.6	4.9 ± 1.5	6.8 ± 2.2

We concluded that the HPLC method used here by including the gradient elution mobile phase application with 5 mM orthophosphoric acid and acetonitrile was a good technique regarding to the detection of HN_2 in plasma. Mass spectrometry application along with HPLC for structural identificaton and approval of HN_2 in eluate which was obtained at the estimated retention time was supportive and integrative for this method used. But, this method should be absolutely confirmed and supported by mass spectrometry whether the retention time was related to HN_2 and derivatization process has been realized as disubstitution.

REFERENCES

1. Cummings J., MacLellan A., Smyth J., 1991. Determination of Reactive Nitrogen Mustard Anticancer Drugs in plasma by HPLC using derivatization. Anal. Chem. 63: 1514-1519.
2. Muller C., Calsou P., 2000. The activity of the DNA-PK complex is determinant in the cellular response to nitrogen mustards. Biochimie. Jan; 82(1): 25-8.
3. Povirk L.F., Shuker D., 1994. DNA damage and mutagenesis induced by nitrogen mustards. Mut. Research. 318: 205-226.
4. Hartley J., Bingham I., 1992. DNA sequence selectivity of guanine N-7 alkylation by nitrogen mustards. Nuc. Acid. Research. 20: 3175-3178.
5. Kallama S., Heminki K., 1986. Stabilities of 7-alkyl-guanosines and 7-deoxyguanosines formed by nitrogen mustard. Chem. Biol. Interact. 70: 289-303.
6. Moller P., Wassermann K., Damgaard J., 2000. Sensitivity to nitrogen mustard relates to the ability of processing DNA damage. Pharm. Toxicol. Apr; 86(4): 169-177.
7. Chen X., Cullinane C., Gray P.J., 1999. DNA damage by nitrogen mustard. Mutat. Res. Sep 15; 445(1): 45-54.
8. Zheng J., Chan K., Muggia F. 1994. Preclinical pharmacokinetics and stability of isophosphoramide mustard. Cancer Chemother. Pharmacol. 33: 391-398.
9. Marrari P., Pianezzola E., Benedetti S., 1996. Determination of tallimustine in human plasma by HPLC. J. Chromatography B. 677: 133-139.
10. White I.N., Suzanger M., Mattocks A.R., 1989. Reduction of nitromin to nitrogen mustard. Carcinogenesis. 10: 2113-2118.
11. Noort D., Hulst A., Trap H., 1997. Synthesis and mass spectrometric identification of the major amino acid adducts formed between sulphur mustard and haemoglobine. 71: 171-178.
12. Skibba J.L., Collins F., 1980. Modification of the 4-(p-nitro-benzyl)pyridine assay for the measurement of melphalan and mechlorethamine in plasma. J. Pharmacol.Methods. 4: 155-163.
13. Taylor J.P., Halprin K.M., Levine V., 1980. Meclorethamine hydrochloride solutions and ointment. Arch. Dermatol. 116: 783-785.
14. Tercel M., Wilson W., Anderson F., 1996. Nitrobenzyl Quaternary Salts as bioreductive prodrugs of the alkylating agent Mechlorethamine. J. Med. Chem. 39: 1084-1094.
15. Vaishnav Y., Swenberg C., 1996. Meclorethamine-induced enhancement of radiation sensitivity of guanine. Int. J. Radiat. Biol. 70 (6): 735-745.
16. Munger D., Sternson L., Repta A., 1977. HPLC analysis of dianhydrogalactitol in plasma by derivatization with sodium diethyldithiocarbamate. J. Chromatography, 143: 375-382.