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COMPARISON OF DETERMINATION OF NITROGEN MUSTARD IN BIOLOGICAL FLUIDS BY USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

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NİTROJEN MUSTARDIN BİYOLOJİK SIVILARDA YÜKSEK BASINÇLI SIVI KROMATOGRAFİSİ (HPLC) VE GAZ KROMATOGRAFİSİ VE KÜTLE SPEKTROMETRİSİ (GC-MS) YÖNTEMLERİYLE ÖLÇÜMLERİNİN KARŞILAŞTIRILMASI

Özet: *Malign hastalıkların kemoterapisinde oldukça yaygın kullanılan ve aynı zamanda bir kimyasal silah olan nitrojen mustardın (HN2) saptama yöntemlerinin standardize edilmesi bu ajana maruz kalanların monitorizasyonu açısından oldukça önemlidir. HN2'nin DNA'nın kovalent modifikasyonu ile etkisini gösterdiği bilinmektedir. Bu çalışmada, Yüksek Basınçlı Sıvı Kromatografisi (HPLC) ve Gaz Kromatografisi-Kütle Spektrometrisi (GC-MS) esasına dayanan iki ölçüm yönteminden bahsedilmektedir. Örneklerin HPLC ve GC-MS sistemine enjekte edilmesinden önce, çeşitli biyolojik sıvılardan, özellikle plazmadan HN2'nin izole edilmesi için Minikolon (Bond-Elut) ekstraksiyon yöntemini kullandık. Sonuçlarda, %80'lik bir geri kazanım ile bu ekstraksiyon yönteminin uygun olduğu görülmektedir. Bu açıdan, derivatizasyon ajanları; HPLC yönteminde kullanılan Diethyldithiocarbamic acid (DDTC2) ve GC-MS yönteminde kullanılan N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA)'nın HN2 ile kombinasyonunda etkin olduğu görülmüştür. Yaklaşık 15. dakikada HN2 - DDTC2 pikini veren ve gradiyent elüsyon tekniğinin kullanıldığı HPLC çalışmasında, bu kompleksin doğrulanması için kütle spektrometrisine ihtiyaç duyulmaktadır. Yöntemin uygulanması açısından GC-MS, HPLC'ye göre daha uygun bulunmuştur. Sonuç olarak; yöntem için gerekli programlar ve türevlendirici ajanlar HN2'nin moleküler yapısına uygun olarak seçilmesi durumunda, HN2'nin biyolojik sıvılarda deteksiyonu için HPLC ve GC-MS tekniklerinin kullanılabileceği saptanmıştır.*

Anahtar Kelimeler: *Nitrojen Mustard, HPLC, GC-MS, Derivatizasyon ajanı.*

Summary: *The standardization of the detection methods of nitrogen mustard (HN2), which is widely used in the chemotherapy of malignant diseases and also as a chemical weapon, is important for people exposed to this chemical compound. It is known that (HN2) acts through covalent modification of DNA. Here is described the comparison of two sophisticated methods based on High Performance Liquid Chromatography (HPLC) and Gas Chromatography-Mass Spectrometry (GC-MS) for the determination of HN2 which had not been performed before by the researchers. We used the minicolumn (Bond-Elut) extraction method for obtaining HN2 from the biological fluids, particularly plasma, before the injection of the sample into HPLC and GC-MS instruments. According to the recovery efficiency results, this extraction method was available taking into consideration the value of 80% recovery. In this connection, the derivatizing agents, Diethyldithiocarbamic acid (DDTC2) used in HPLC method and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) used in GC-MS method, were effective agents for easily combination with HN2. In HPLC application, we need, in addition, mass spectrometry for confirmation of the HN2 - DDTC2 complex compound having retention time of 15 min. Moreover, HPLC running program so called "gradient elution" for the mobile phase application gave stable HN2 - DDTC2 peak at 15th min*

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approximately. When compared to HPLC, GC-MS was easier than HPLC in terms of the program application. We conclude that HPLC and GC-MS methods should be prefered for the determination of HN2 available in biological fluids, if the running programs and derivatizing agents could be elected in accordance with HN2 molecular structure.

Key Words: *Nitrogen Mustard, HPLC, GC-MS, Derivatization agents.*

INTRODUCTION

The nitrogen mustard (HN2) [mechlorethamine; 2 - chloro - N - (2 chloroethyl) – N –methyl ethanamine], is one of the oldest anticancer agents and is still extensively used in the chemotheraphy of malignant diseases (1). Nitrogen mustard is a bifunctional alkylating agent that is thought to act via covalent modification of DNA (2,3,4). Early studies on the reaction of HN^2 with DNA and RNA provided evidence that a monoalkylation product and a cross-link were formed at N-7 of guanine. HN_2 has also been considered as a chemical weapon being highly unstable. It has the greatest blistering potential in a vapour form affecting especially skin, eyes and respiratory tract (5,6). Following ingestion or systemic absorption, HN_2 causes inhibition of cell mitosis resulting in depression of the blood-forming mechanism and injury to other tissues (7). In order to analyze HN_2, a number of chemical compounds, such as Diethyldithiocarbamic acid (DDTC) and N-methyl-N-trimethylsilyltriflouroacetamide (MSTFA), have been used succesfully in terms of trapping HN2 in the advanced methods (8,9,10) (Figure 1). In this study, it was targeted to establish a sensitive determination method of HN2 comparing to HPLC and GC-MS tecniques.

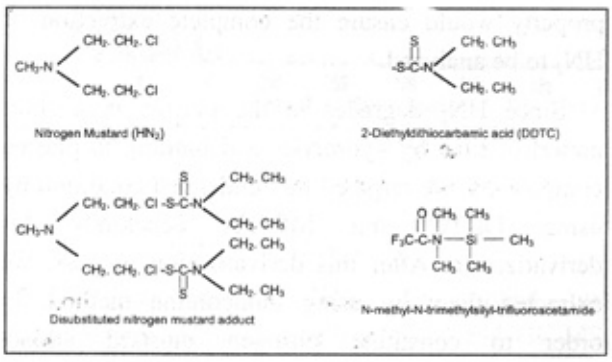

Figure 1. Molecular structures of nitrogen mustard and derivatization agents

MATERIALS AND METHOD

a. Materials: Diethyldithiocarbamic acid – sodium salt, N – methyl – N –trimethylsilyl trifluoroacetamide, Mechlorethamine hydrochloride (Nitrogen mustard) and "Bond Elut" minicolumns (SUPELCO®) which have 4 ml capacity were purchased from SIGMA Chemical Co. Methanol (106018), Acetonitrile (115500), Hexane (104391), Chloroform (102444), isopropanol were bought from Merck Co. In this study, deionized and bidistilled water was used.

b. Precautionary Steps: All steps of HN_2 preparation were performed in a permanently running Laminar flow cabinet (fume hood) (125 feet/min, Israel) for protecting ourselves. All contaminated glassware and disposable materials were neutralized with Natrium hydroxyde (5M) / Ethanol (1/10, v/v) solution for at least 24 h. While working, the other required measures were all taken (11,12).

c. Derivatization And Sample Preparation: For preparing samples to be applied to both HPLC and GC-MS, the plasma containing nitrogen mustard of different concentrations was used. These samples were derivatized by both diethyldithiocarbamic acid-sodium salt and N-methyl-N-trimethylsilyltrifluoroacetamide separately, after they were assigned into two groups. For derivatization, 50 μl of DDTC freshly prepared in 0.1 N Natrium hydroxide and 50 μl MSTFA were added into each group of plasma samples. Then, specimens were incubated for 30 min at 37 °C in water bath (13,14). After derivatization, specimens were extracted by using minicolumns. During extractions, firstly. minicolumns were activated with 2 ml of water and then 2 ml of sample was loaded into each column. After washing with 5 ml of water, they were kept at room temperature at dark. Adduct compounds were eluted with 2 ml

acetonitrile which would be evaporated by a centrifugal vacuum evaporator (Speed Vac-Plus-SC 110 A) mounted Refrigerated Vapour in 200 µl of methanol.

d. High Performance Liquid Chromatography: In this study, a VARIAN 5000 model HPLC system equipped with a Rheodyne 7125 sample injector (20 µl loop) connected to an optical detector (UV-VIS 200, LINEAR Corp. Reno, NU, USA) was used. The column used here was (4 µm Supersphere 100, RP-18, 250x4 mm i.d., Merck, Darmstadt, Germany) with a guard column packed with the same matrix. We also used the optical detector and applied the mobile phase which involves a gradient including mobile phase A (50 mM o-phosphoric acid) and mobile phase B (acetonitrile). The initial rate of the gradient was 30 % B and 70 % A. Mobile phase B was maintained for 4 min and then increased linearly to 100 % B within 13 min, returning back to 30 % B by 20 min running lasted for 30 min. Mobile phase solutions were degassed by vacuum.

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. The other operating conditions were; Emission current; 1 mA; electron energy 70 eV; ion source temperature, 250 ^{0}C; ion accelaration voltage, 3-5 Kv .

f. Gas Chromatography-Mass Spectrometry: Hewlett-Packard 5971-GCD Plus coupled to a Mass spectrometer via an glass jet seperator was used in this study. The column used here was a high performance capillary column (Hewlett-Packard) with HP-1 (crosslinked methyl siloxane), 30 m x 0.25 mm I.D., film thickness 0.25 µm. Helium was as carrier gas at a flow rate of 0.7 mL /min. Mass range of the detector was 10-450 m/z. The oven temperature program was applied as following: Initial temperature was 40 ^{0}C for 2.0 min, then increased at 10 ^{0}C/min to a final temperature 280 ^{0}C and held for 10 min. This running program consisted 250 ^{0}C as temperature of the interface, 200 ^{0}C for ion source temperature and 250 ^{0}C for injector temperature. Moreover, we applied the splitless injection as 1 µL.

RESULTS AND DISCUSSION

The nitrogen mustard (HN_2) is not only one of the chemical weapons used, but also the anticancer drug used widely in malignant diseases. Accordingly, it is important to detect it in biological fluids and contaminated environmental materials such as soil, water, clothes, etc. In the clinical studies for observing patients, determination of the level of HN2 in the blood and urine is necessary in terms of the stability of the patient. For this reason, we applied two developed and sophisticated instruments, High Performance Liquid Chromatography (HPLC) and Gas Chromatography-Mass Spectrometry (GC-MS), in addition to mass spectrometry providing information related to the molecular structure of HN_2 (14,16,17).

In this study, our aim was to set up an effective method determining the HN_2 spiked into plasma by utilizing HPLC and GC-MS. One of the significant processes in this work is the extraction of the HN_2 from blood samples effectively. For this stage, we used minicolumns containing silica gel. According to the papers published (18, 19, 20), endogen components of plasma which could interfere the precise identification of HN_2 are eliminated by these minicolumns having capacity of 2-4 ml. Also this property would ensure the complete extraction of HN_2 to be analyzed.

Since HN_2 degrades in the plasma in a short period of time by hydrolysis and binding to plasma components, we targeted this undesired condition by using DDTC and MSTFA seperately for derivatization. After this derivatization process, we extracted them by using minicolumn method. In order to constitute nitrogen mustard adduct disubstituted by DDTC, 100 µl DDTC freshly

prepared in 0.1 N natrium hydroxide was added into 1ml plasma samples containing HN_2 and then incubated 30 min at 37 C. Our aim here was to form disubstituted derivatives of HN_2 which had UV spectrum at 276 nm. At the end of this incubation, we checked whether disubstituted nitrogen mustard adducts (HN_2 - $DDTC_2$) had occurred regarding molecular structure scanned by using mass spectrometry – LKB 2091. Mass spectrum of molecular ion MH+, m/z 386 obtained would indicate that disubstituted HN_2 adducts have taken place in the end of the derivatization process (Figure 2). To perform MSTFA derivatization, we also followed the same derivatization procedure used with DDTC. Residues containing HN_2 - $DDTC_2$ and HN_2 – MSTFA separately were dissolved with 200 μl methanol. These derivatizing agents are the most proposed compounds for the derivatization of HN_2 in accordance with previously published literatures (10, 20, 21, 22). We also would approve that both agents are effective for this procedure based on mass spectrum of molecular structure of derivatizing complex obtained by mass spectrometry.

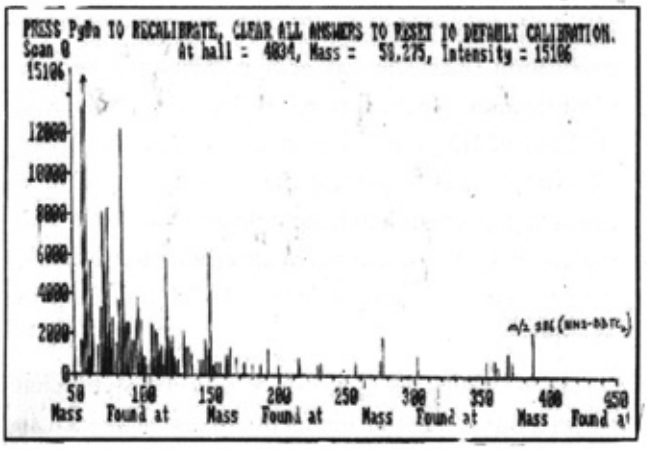

Figure 2. Structural identification of nitrogen mustard-DDTC2 derivatives has been evaluated by mass spectrometry, and molecular ions and electron impact mass spectra of the derivatives are illustrated in figure above.

To determine the HN_2, we applied two measurement techniques called HPLC and GC-MS. In HPLC study, we introduced HN_2 in the form of HN_2 - $DDTC_2$ complex to HPLC system including optical detector and running with gradient elution mobile phases application which we accepted as an available HPLC running program regarding the determination of HN_2 . As a mobile phase, 5 mM orthophosphoric acid and acetonitrile were used. By using this program, we found that the HN_2 - $DDTC_2$ compound had the retention time (t_R) at 15th min. Having collected the elution material formed at 15th min, we analyzed the sample eluted with respect to the molecular structure by using mass spectrometer in order to approve that the HN_2 - $DDTC_2$ complex had retention time at approximately 15th min. At the end of this evaluation, we agreed that the peak observed at the 15th min in a chromatographic run was related to HN^2 - $DDTC^2$ (Figure 3).

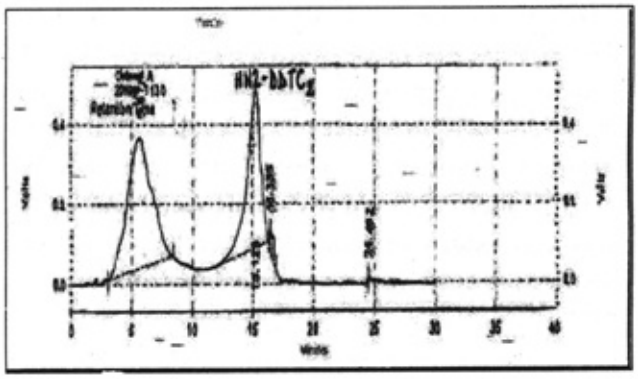

Figure 3. Chromatogram of nitrogen mustard (HN_2) after derivatization with diethyldithiocarbamic acid in 0.1 N NaOH. Disubstituted nitrogen mustard has been determined to have 15.123 min. of retention time.

In GC-MS study for the determination of HN^2, we followed the same extraction method for plasma exposed to HN^2 with different concentrations (18, 19). Derivatization of HN^2 with 50 μl of MSTFA was performed prior to minicolumn extraction and then the sample eluted by acetonitrile was evaporated to dryness in a centrifugal vacuum evaporator. After residue was dissolved in methanol, sample was injected to GC-MS system within a short time. Based on the evaluation of GC-MS chromatograms obtained, the recovery efficiencies of minicolumn extraction were found as 80 % of expected (spiked into plasma) values. For precise setting of GC-MS

running program, several trials were applied and examined. The available program for the determination was chosen one which had the oven temperature beginning with 40 C for 2.0 min and increasing to final level at 280 ^{0}C. Splitless injections (1 μl) were given with the injector temperature of 250 C and split delay of 0.5 min. During GC-MS process approximately at 11.17 min, we obtained the mass spectrum related to 2-chloro-N-(2-chloroethyl)-N-methyl- which is estimated as a large fragment of HN_2, and trimethylsilyl ester which is also a large fragment of MSTFA observed at 11.44 min. That the mass spectrum of both large fragments were seen at the approximation of 11th min could mean that the spectrum of HN_2 – MSTFA complex compound would be obtained at a definite time, as explained above. These mass spectra related to HN_2 – MSTFA complex are shown in Figure 4 and 5 where the chromatograms are indicated. According to obtained data, a procedure based on trimethylsilyl derivatization followed by minicolumn extraction was also agreed as a developed technique for the verification of HN_2 and its degradation products.

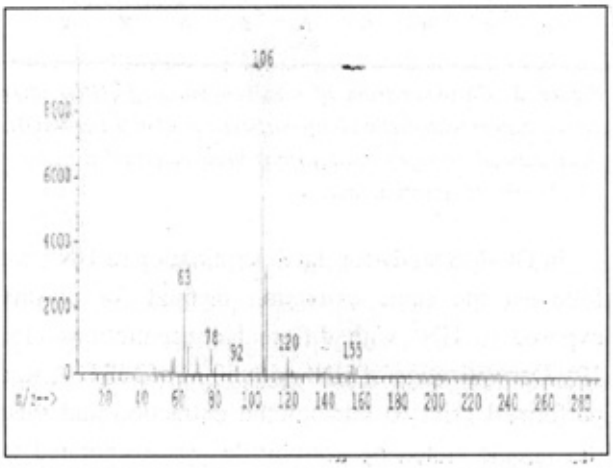

Figure 4. *Mass spectrum of nitrogen mustard derivatized by N-methyl-N-trimethylsilyltrifluoroacetamide has been obtained at approximately 11th min as 2-chloro-N-(2-chloroethyl)-N-methyl compound.*

We also applied the derivatives of HN_2 with DDTC and MSTFA containing different concentrations of HN_2 which were spiked into plasma fixed values of derivatizing agents. Taking into consideration the losses of compounds during extraction, 80 % recovery efficiencies found for them dominantly meant that the minicolumn procedure was an available method for obtaining HN_2 form biological fluids.

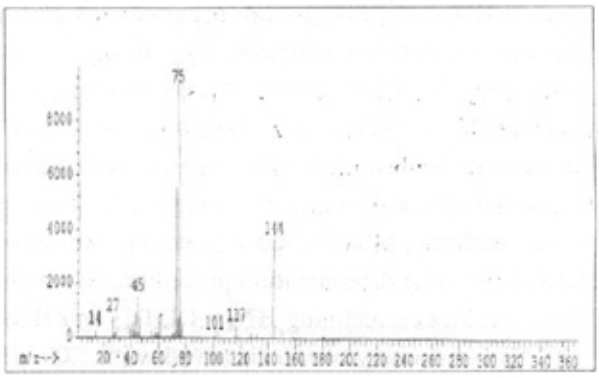

Figure 5. *Mass spectrum of N-methyl-N-trimethylsilyltrifluoroacetamide used in derivatization of nitrogen mustard is inidcated at 11.44th min.*

We needed mass spectrometry in HPLC method for the confirmation of HN_2 – DDTC whether the retention time in 15th min was related to this complex. However, due to GC-MS technique having MS and library, we evaluated that the GC-MS application had the advantage regarding to its identification. In addition, taking the molecular structure of HN_2 into account, we would rather apply GC-MS in order to get a stable spectrum at a certain time.On the other hand, no related study was met regarding to the quantitative determination of HN_2 with respect to the comparison of HPLC and GC-MS methods.

As a result, as seen in Figure 6, significant correlation, slope and intercept values were seen in accordance with each of the methods (R2= 0.9957, slope= 1.0786, intercept =-0.0077).

It is concluded that especially in biological fluids containing nitrogen mustard both as drug or a toxic chemical, determination of HN_2 and its degradation products is possible by using HPLC and particularly GC-MS methods.

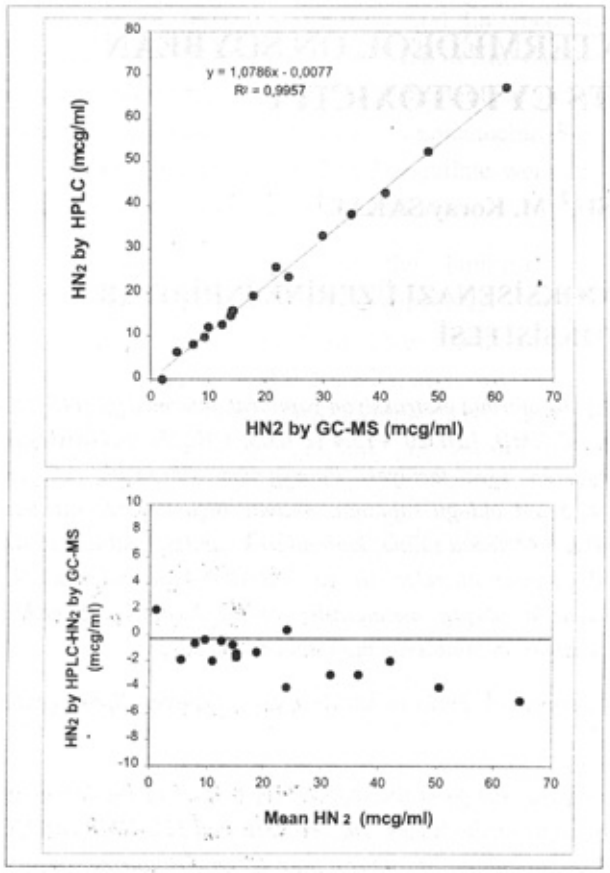

Figure 6: Comparison of modified HPLC and GCMS method. Regression analysis of HN_2 measurement by HPLC versus GCMS and the difference between two methods is plotted against the mean results.

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