GC/MS Determination of N-butyl-N-(3-carboxypropyl) Nitrosamine (BCPN) in Bladder Cancers – The Skewed Molecular Interaction Caused Retention Time Shift

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Abstract

N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) has been widely used in rodents as an invaluable experimental tool for investigation of bladder cancer (BCA). The urinary level of its metabolite, N-butyl-N-(3-carboxypropyl) nitrosamine (BCPN) was reported to be a very reliable predictive parameter of BCA. However, in determination of the urinary BCPN we found the retention time (tR) of BCPN was randomly damping. The tR values of the authentic BCPN at 5, 10, 20, 50, and 100 ppm were 28.48, 27.59, 27.43, 28.00, and 28.32 min comparing with 28.23 min of the urinary BCPN in HPLC analysis, similarly, 17.30 min for the urinary and the 18.00 min for the authentic BCPN in GC/MS analysis. To interpret such a damping, we theoretically proposed that a certain transient skewed molecular interaction could occur during the chromatographic separation, which would cause a certain degree of fluctuation on the tR of target molecules. Conclusively, the retention time of a chemical is not a definite value as often considered in HPLC and GC/MS analyses. In reality it fluctuates mainly upon the interaction among a cluster of coexisting molecules, in particular, when operated at higher concentrations.

Keywords: N-butyl-N-(4-hydroxybutyl) nitrosamine; N-butyl-N-(3-carboxypropyl) nitrosamine; bladder cancer; HPLC; GC/MS

Introduction

As N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) can induce bladder cancer very similar to transitional cell carcinoma in patients in both kinetics and histologic features, hence has been widely used in animal modeling [1–3].

Currently, cigarette smoking has been considered to be an epidemiological major risk factor of developing bladder cancer in industrialized countries. While N-nitroso compounds (NOCs) have been proposed as etiologic agents of bladder cancer associated with Schistosomiasis [1–3], N-nitrosodibutylamine (NDBA) is the first chemical identified as a rat bladder carcinogen that has been detected in tobacco smoke. Although at low concentrations, it is considered practically carcinogenic to humans [4]. NDBA is metabolized mainly in liver. Its tumor inducing capability in rat bladders seems to depend on the formation of two ω-oxidized metabolites, N-nitrosobutyl (4-hydroxybutyl) amine (BBN) and its proximate carcinogen N-nitrosobutyl (3-carboxypropyl) amine (BCPN) [5]. Physiopathologically, BBN-induced urinary bladder carcinogenesis in rodents is an excellent model system to understand the carcinogenic mechanisms by NOCs [6].

Several lines of epidemiologic and experimental evidence suggest that a decreased expression in carcinogen-detoxifying enzymes, such as N-acetyl transferase 2 [7,8], glutathione S-transferase (GST) M1 [7,9], NAD (P) H quinone oxidoreductase [10], and UDP-glucuronosyltransfase (UGT) 1A [11], is associated with urinary bladder cancer. BCPN, the major urinary metabolite of BBN, has been shown to have carcinogenic effects on urothelial cells [12,13]. Following α-hydroxylation, BCPN and BBN are chemically cleaved to their corresponding alkylcarbionium ion that binds covalently to DNA and enhances carcinogenesis [14] (see Appendix Figure A1 [15]).

Theoretical

To discuss the molecular interaction in a binary component system, we have first to introduce the solubility parameter, δ which is defined as

\[ \delta = \frac{\Delta E}{V} \]

where \( \Delta E \) is the energy of vaporization to gas at zero pressure (i.e. infinite separation of the molecules); \( V \) is the molar volume; while the ratio \( \Delta E/V \) is always defined as the cohesive energy density (CED).

Alternatively, the Flory-Huggins equation (Eq. 2) [16] is the very

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popularly cited equation to discuss the interaction between (or among) the molecules by considering the difference of solubility parameters.

\[ X_{i,j} = \beta_{i,j} + \left( \frac{\Delta H_i}{RT} \right) (\delta_i - \delta_j)^2 \]  (2)

Here the symbol \( X_{i,j} \) on the left hand side denotes the Flory-Huggins molecular interaction parameter of a solvent with the polymer; \( \Delta H \) means the enthalpy change of mixing; \( k \) is the Boltzmann constant; \( T \) is the absolute temperature in Kelvin scale at which the experimental data are developed; \( N_i \) is the number of molecules of species \( i \); \( v_i \) is the molar volume of molecular species \( i \); \( v \) is the molar volume of molecular species \( j \); \( R \) is the gas constant; \( \delta_i \) and \( \delta_j \) denote the solubility parameters of molecular species \( i \) and \( j \), respectively. And the parameter \( \beta_{i,j} \) is the interaction parameter under conditions completely soluble or miscible, i.e. under condition \( \delta_i = \delta_j \) (Eq. 2). The best condition for solute-polymer interacting pair is \( \beta_{i,j} = 0.35 \) [16]. Thus the smaller the difference between the solubility parameters, the more likely one chemical could be dissolved in or miscible with the counterpart solute.

Assuming the Eq. 2 is applicable to all situations dealing with the molecular interaction, we have for a cluster of molecular event the relationship

\[ \Sigma X_{i,j} = \Sigma \left( \frac{\Delta H_i}{RT} \right) (\delta_i - \delta_j)^2 \]  (3)

Which denotes that the centered target molecule \( i \) act with its surrounding molecules (\( j = 1, 2, 3, \ldots \)). Obviously, here the vector effect has been omitted for simplification. Consequently, by considering the vector factor, the overall interaction parameter is arbitrarily modified as \( \Sigma X_{i,j}' \) that actually has involved the concept of vector sum.

\[ \Sigma X_{i,j}' = \Sigma \left( \frac{\Delta H_i}{RT} \right) (\delta_i - \delta_j)^2 \]  (4)

However, in chromatographic analysis, the true situation is more complicate. Using the inverse gas chromatography (IGC) measurement, King obtained the solubility parameter of the solutes [17]. Such a deduction requires the computation of intermediate parameters, namely the solute-solvent interaction parameter, \( X_{i,j} \) which is directly derivable from the specific retention volumes measured in the IGC experiment [17]. Specific retention volumes, \( V_i \), for the chosen solutes in the stationary phase can be calculated from the retention time data as previously described by King et al. [18]. These retention volumes are then utilized to compute the interaction parameter, \( X_{i,j} \), between the solute \( i \) and solute \( j \) according to Eq. 5 [19]. Details of the computation of the specific retention volumes are given in standard texts [20] and will not be repeated here.

\[ X_{i,j} = \left[ \frac{[\Delta H_i]}{[RT]} \right] - \left[ \frac{1}{[RT]} \right] \]  (5)

where \( R \) = 8.31 \times 10^{-2} \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1} \)

\( v_i \) = specific retention volumes

\( M_i \) = molecular weight of the solute \( i \)

\( B_i \) = second pure virial coefficient of the solute \( i \) at \( T \)

\( c_i \) = vapor pressure of solute \( i \) at column temperature, \( T \)

\( V_i \) = molar volume of solute \( i \) at \( T \)

\( T \) = column temperature

\( v_i \) = specific volume of the solute \( i \)

\( M_i \) = molecular weight of the solute \( i \)

It should be noted when IGC is used to determine \( X \) parameters for polymer(solvent) interaction, that the last two terms in Equation 5 are often omitted, since they contribute negligibly to \( X \) for the case of an infinitely high molecular weight polymer [17]. This is not the case, however, for a solute having a low molecular weight such as BBN and BCPN, these terms should be computed and used in the calculation of \( X \). To compute the second virial coefficient of the solute, the well-known relationship developed by McGlashan and Potter [21] as given in Eq. 6 can be applied.

\[ B_i V_i = 0.430 - 0.866(T/T_c) - 0.694(T/T_c)^2 - 0.0375 (n - 1)(T/T_c)^4 \]  (6)

where \( T_c \) = critical temperature of the solute

\( V_i \) = critical volume of the solute

\( n \) = hypothetical number of carbon atoms for a given solute that yields a Po, equivalent to that of a corresponding n-alkane solute.

The parameter \( n \) can be calculated according to the procedure of Guggenheim and wormald [22] and King et al. [18].

Substitution of the values of \( B_i \) obtained from Eq. 6 into Eq. 5 yields \( c \) the interaction parameters \( X_{i,j} \) for each pair of solutes (Eq. 6). Substitution of the value of \( X_{i,j} \) into Eq. 3 in turn leads to the solubility parameter by comparing with the solubility of a reference compound. Obviously, when operated at very low concentration, each molecule would acts freely without any interacting interference from the surrounding molecules. However, as the HPLC or GC operation concentration is increased, the interaction of the surrounding molecules would be also steadily increased. Thus the resultant molecular interaction parameters \( \Sigma X_{i,j} \) could appear as either repelling or dragging (Figure 1), where \( C_i \) is the centered target molecule, \( C_i (j = 2, 3, \ldots) \) are the surrounding molecules.

As similarly treated in Eq. 4, on consideration of the vector factor we have from Eq. 5 the overall effective interaction parameter, \( \Sigma X_{i,j}' \), along the separation column resulting from a cluster of molecular interactions.

![Figure 1: The vector sum of different interaction parameter results in different net effect acting as either repelling (upper) or dragging (lower) on the target molecule CI.](image)

The target molecule \( C_i \) to be identified is adsorbed by the stationary phase (e.g. silica gel column). Other coexisting molecules designated as \( C_j (j = 2, 3, 4, 6, 7, \ldots) \) are interacting with this centered molecule \( C_i \) in different degree of force. The intermolecular action depends on each individual solubility parameter, \( \delta_i \) and \( \delta_j \) (\( j = 2, 3, 4, 6, 7, \ldots \)). The resultant vector sum can be either repelling (upper) or dragging (lower). In a system of infinitely dilution these intermolecular forces can be neglected. As can be expected, the higher concentration of sample would exhibit higher degree of interaction to result in more serious shit of the retention time.
Experimental

Chemicals and materials

Ethyl acetate was manufactured by E. Merck Co. (Germany). N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) and N-butyl-N-(3-carboxypropyl) nitrosamine (BCPN, 96% in purity) were provided by Kasei Industrial Co. (Tokyo, Japan). Methanol was purchased from Sigma (Mo., USA). Hydrochloric acid was product of Wako Pure Chemicals Inc. (Tokyo, Japan). Micropore (0.2 µm) was a product of Pall Co. (USA).

HPLC analysis of N-butyl-N-(3-carboxypropyl) nitrosamine (BCPN)

The HPLC analysis of BCPN was carried out according to Wada et al. [24]. The HPLC was Hitachi HPLC (installed with an L-2130 pump and an L-2480 FL Detector (Hitachi HiTech, Tokyo, Japan). The high speed centrifuge, Mode-3740, was provided by Kubota Co. (Japan). The analytical column used was RP18, Supelco (USA). The ultrasonicator DC200H was purchased from Delta Chemical Sejahtea (Jakata-Indonesia).

Preparation of BCPN standard solution

0.01 g of authentic N-butyl-N-(3-carboxypropyl) nitrosamine (BCPN) was accurately measured and dissolved in ddw to make a total volume of 10 mL. This standard solution containing 1000 ppm of BCPN was used as the stock solution of BCPN and kept at 4°C in the dark for further use. The stock solution was successively diluted with ddw to concentrations of 10, 20, 30, 50, and 100 ppm, respectively.

Preparation of fluorescence derivative of BCPN with anthryl diazomethane (ADAM)

Standard solution of BCPN (0.5 mL) was accurately measured and transferred into a 5 mL tube. To which 100 µL of HCl (2 N) and 0.5 mL of ethyl acetate were added and agitated vigorously for 1 min. The sample was centrifuged at 3000×g for 15 min. The supernatant layer was separated. The supernatant (0.5 mL) was measured and dried under nitrogen blow. To the residue 0.5 mL of ADAM solution (800 µg/mL in ethyl acetate) was added. The mixture was left for 1 h to facilitate the reaction. HPLC analysis was carried out. The amount of sample injected was 0.3 µL each time. The column type was Hitachi RPC18 (ℓ=150 mm, id=2.0 mm). The mobile phase was a mixture of methanol: water (80:20 v/v) operated at a flow rate of 0.6 mL/min. The fluorescence was monitored with a fluorescence detector (FL type L-2480) at λEc = 365 nm; and λEm=430 nm. The arrows indicate the retention time of the BCPN derivative. The correlation equation obtained was Y=266716x+2E+6 (R^2=0.9441), where Y was the peak area and x was the concentration of BCPN (ppm).

Determination of urinary BCPN

The urinary level of BCPN was determined according to Ozaki et al. [23] with slight modification. For mass treatment, a precolumn was suggested to be used for purification. Briefly, the separation was performed with a mobile phase consisting of a mixture of acetonitrile and sodium acetate buffer (20 mmol/L, pH 4.5) (3:7 v/v). The flow rate was set at 1 mL/min. The fraction that appeared with a retention time of 7.8 min (BCPN) was collected. The recovery yield of BCPN from the urine was about 60% under our assay conditions. The product BCPN obtained was assayed similarly as mentioned in the above. Alternatively, for simplicity, 0.1 mL of urine sample was diluted to 0.5 mL with distilled water. A 3.3-µL aliquot of 12 mol/L HCl was added. The sample was repeatedly extracted with 0.5 mL of ethyl acetate for...
three times. The organic layers were collected after centrifugation for 5 minutes at 10,000×g and dried in a high speed vacuum concentrator with a cooling trap maintained at 30°C. The residue was dissolved in ethyl acetate and spotted onto a silica gel 70 F254 precoated plate (Wako Pure Chemical Co., Osaka, Japan). The plates were developed with chloroform/methanol/acetic acid (18:1:1, v/v) in the dark. The bands corresponding to BBN or BCPN (The Rf values reported to be 0.68 to 0.72) were scraped off and extracted out off the silica gel with 4 mL of acetone. The extract was concentrated in a high speed vacuum concentrator. The residual viscous portion was redissolved in acetonitrile to a final volume of 0.2 mL and filtered through a MilliPore RC4 filter (0.2-µm pore size; Sartorius, Göttingen, Germany). The filtrate was subjected to HPLC analysis at 239 nm for determination of the urinary BCPN level using Shimazu LC9A HPLC (Shimazu, Kyoto, Japan) installed with a Finepak SIL C18 column (Jasco, Tokyo, Japan; 5 µm, id = 4.6 mm).

### Gas Chromatographic analysis of N-butyl-N-(3-carboxypropyl) nitrosamine (BCPN)

The determination of BCPN with gas chromatography was conducted according to Ozaki et al. [23] and Wada et al. [24] with a slight modification. Briefly, the urine sample was treated with a primary separation column to exclude all possible contaminants. The purified product was dried under nitrogen flow. 10 mg of the residue was methyalted with a mixture of 10% of sulfuric acid and 6 mL of methanol. The reaction mixture was frequently agitated at ambient temperature for 5-6 h to facilitate the reaction. 5 mL of water was added to terminate the reaction. To the mixture 6 mL of chloroform and anhydrous sodium sulfate was added. After agitated vigorously for 2 min, the mixture was left to stand to facilitate the separation. The supernatant was measured and subjected to GC/MS analysis.

### Condition of GC/MS operation

The GC/MS GC HP 6890 attached with HP5973MSD detector and a capillary column DB-5 (ℓ= 60 cm, i.d= 0.25 mm, membrane thickness, 0.25 μm) was used. The injection port and the detector were maintained at 270 and 280ºC, respectively. The volume of supernatant applied was 1 μL. The ionization potential used was 70 eV where the temperature of ion source was held at 230ºC. The temperature of column was programmed as: initially at 40ºC for 10 min, then programmed at an elevation rate of 2 ºC/min until up to 280 ºC and maintained at which for 30 min. The mobile phase was his gas operated at a flow rate of 1 mL/min.

### Results and Discussion

Figure 2 indicate the retention time (tR) of reference BCPN at different concentration. As seen, the tR values of reference BCPN fluctuated randomly at 28.48, 27.59, 27.43, 28.00, 28.33 min, respectively, for 5, 10, 20, 50 and 100 ppm of BCPN. From which a correlation was achieved as Y= 266716 X+2.0×10^6 (R^2=0.9441), where Y is the peak area and X is the concentration of BCPN (ppm) (not shown), whence the content of BCPN in urine specimen having a tR = 28.23 min was calculated to be 12.8 ppm. Moreover, taking the median concentration 50 ppm as a reference point, the tR was also found to have shifted from 28.00 min for reference BCPN (50 ppm) (Figure 3a) to 28.23 min of urinary BCPN (Figure 3b).

Similar phenomenon was also noted in GC/MS analysis. The tR value was 18.00 min for the reference BCPN, while the urinary sample BCPN shifted to 17.30±0.09 min for the reference BCPN (50 ppm) (Figure 3a) to 17.30±0.09 min of urinary BCPN (Figure 3b).

### Conclusion

Conclusively, the retention time is not absolutely a definite value as often considered in the HPLC or a GC/MS analysis. In reality it may fluctuate depending mainly upon the coexisting molecular interaction,

Figure 4: GC/MS analysis of BCPN (N-butyl-N-(3-carboxypropyl) nitrosamine). Standard BCPN ($t_R = 18.00$ min) (a), and BCPN in urine ($t_R = 17.30$ min)(b).‡

‡The pretreatment method for GC analysis is presented in the text.
in particular at a high concentration operation, underlying several causes of shifting which can be minimized but not abolished by careful manipulation of the postulates as mentioned.

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