Col Liquid Chromatography

Method development for the acquisition of adsorption isotherm of ion pair reagents Tributylamine and Triethylamine in ion pair chromatography

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A B S T R A C T

Tributylamine (TBuA) and triethylamine (TETA) are the most commonly used ion pair reagents in ion pair chromatography especially for the analysis of oligonucleotides. In order to improve the understanding of the retention and separation mechanism of oligonucleotides in ion pair chromatography, it is important to understand the retention mechanism and the nature of interaction of these ion pair reagents with the stationary phase in the chromatographic column. Adsorption isotherm is helpful in evaluating such interactions, and subsequently predicting the retention mechanism. Alkylamines are very polar molecules which lack suitable chromophore and are commonly present in charged forms. Therefore, their determination and the subsequent acquisition of their adsorption isotherms using traditional liquid chromatography is very difficult. In this study, we first developed an analytical method for the determination of TBuA and TETA in a typical chromatographic mobile phase (acetonitrile-water) and then used the same method to acquire the adsorption isotherms for tributylammonium acetate (TBuAA) and triethylammonium acetate (TETAA). This method started with the conversion of the alkylammonium ions to free neutral forms by treating the sample with a strong base, followed by pentane-mediated extraction and finally the analysis of the extracts using gas chromatography-flame ionization detector (GC-FID). This three-step method was validated for parameters like range, linearity, intra-day and inter-day precision and accuracy, limit of detection and limit of quantitation. For the adsorption isotherms, the C18 column was first equilibrated with the solutions having different concentrations of alkylammonium ions and then stripped with eluent devoid of alkylammonium ions. Several stripping eluents were investigated and it was discovered that the eluent requirement could be decreased by the addition of sodium chloride. The effluents from the stripping phase were collected and analyzed using the developed analytical method to acquire the adsorption data. Under the investigated conditions, adsorption of TBuAA and TETAA showed type III and type I isotherm behavior respectively.

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1. Introduction

Oligonucleotides have emerged as one of the most valuable families of therapeutic agents for a wide range of diseases including cancer (liver, breast, and brain tumour), viral infections (Cytomegalovirus, Human immunodeficiency virus, hepatitis B and C, Influenza, etc.), neurodegenerative diseases, obesity, cardiovascular disorders and genetic diseases like spinal muscular atrophy, X-linked agammaglobulinemia and familial hypercholesterolemia [1,2]. Ion pair-reversed phase (IP-RP) chromatography is the most widely used analytical technique and has become a gold standard method for the analysis of oligonucleotides [3–5]. Oligonucleotides are polar, charged molecules with multiple negative charges. This implies that ion pair reagents (IPRs) have to be used in the mobile phase to retain them in the stationary phase of the reversed phase liquid chromatography. Historically, the IPRs have consisted of combinations of alkylamines with acetate as counter-ions. For example, triethylamine (TETA) and tributylamine (TBuA) are among the most commonly-used IPRs in IP-RP chromatography for analysis of oligonucleotides [5–7]. At the time of writing, in many analytical applications, acetate counter-ions have been replaced by 1, 1, 1, 3, 3, 3-hexafluoropropan-2-ol (HFIP) to decrease ion suppression during the mass spectrometry analysis [8].

The retention mechanism in IP-RP chromatography is very complex and governed by a number of factors [9,10]. In one of the models, the IPR and the oppositely-charged solute molecule

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Abreviations: GC, Gas chromatography; LC, Liquid chromatography.
interact with each other electrostatically in the mobile phase to form a neutral complex which then interacts hydrophobically with the nonpolar stationary phase [11–13]. In another model, the IPR in the mobile phase first gets adsorbed to the nonpolar stationary phase, through the hydrophobic interaction of the alkyl side chain of the IPR. This creates a dynamic ion exchange layer on the surface of the stationary phase which behaves like an ion-exchanger for the oppositely-charged solute molecules [13,14]. The dynamic complex exchange model was proposed by W. R. Melander and C. Horvath to account for the lack of fit of the aforesaid two models (or a combination of the same) to the experimental data [15]. According to the Melander-Horvath model, both ion pair formation in the mobile phase and the adsorption of IPR to the stationary phase, occur simultaneously and independently of each other. Then the IPR interacts with the adsorbed IPR to form a ternary complex on the surface which subsequently decomposes, releasing the already bound IPR in the process [15]. Another model called the ion-interaction model assumes that the retention is a dynamic equilibrium, affected not only by the electrostatic interaction between the IPR and solutes, but also by other forces involved among the IPR, solute, eluent and the stationary phase [10]. More complex theoretical models like the liquid partition double layer model and the diffuse layer ion-exchange double layer model exist for the explanation of retention in IP-RP chromatography[16,17].

Due to lack of complete understanding, it has not been possible to predict which retention mechanism is followed under the given circumstances. The nature of retention may change from one to the other as the chromatographic conditions change [11]. Irrespective of which retention mechanism is operational, the role of IPRs is vital in IP-RP chromatography. Therefore, it is crucial to understand the interaction of IPRs with the stationary phase under the conditions used in IP-RP chromatography. Adsorption isotherm is the key for understanding the nature of interaction between the surface of the stationary phase and the solute in the mobile phase, and subsequently predicting the retention mechanism [18]. Recently, Leško and colleagues have shown that the adsorption of IPR is also crucial for the accurate modeling of preparative elution profiles [19]. An adsorption isotherm describes the concentrations of solute in the stationary phase and mobile phase at equilibrium at a given temperature [18]. Several different adsorption isotherm models can be used to model the processes, and they are grouped according to their shapes [20]. The most common ones are the type I isotherms (Langmuir, for instance), which are convex; the amount of the adsorbed solute approaches a limiting value as the solute concentration increases. This model accounts for adsorption processes where only monolayers are formed. The type III isotherms [some cases of Brunauer-Emmett-Teller (BET) or anti-Langmuir, for example] are concave and the amount of adsorbed solute does not have a limited value. In this case, no identifiable monolayer is formed, the adsorbed solutes rather being clustered on the surface. The adsorption isotherms for tetraalkylammonium ions on reversed phase columns have been reported extensively under diverse chromatographic conditions [21–25] and these are type I isotherms. However, there is a paucity of information about the adsorption isotherms of trialkylammonium ions, and to the best of our knowledge, there are no published data in the scientific literature.

The determination, and thereby the acquisition of adsorption isotherm of alkylamines or alkylammonium ions directly through liquid chromatography (LC) is very difficult because they lack a suitable chromophore and produce peak tailing or peak shapes with asymmetry due to their very polar and charged nature [26–28]. Bartha and Vigh have reported the acquisition of adsorption isotherm for tetrabutylammonium ions through breakthrough curve using refractive index (RI) detector [23]. However, due to the insensitive nature of the RI-detector, the adsorption isotherm can only be acquired for relatively high concentrations. On the other hand, several researchers [21,22,24,29] have reported the acquisition of the adsorption isotherm for tetraalkylammonium ions using batch method, in which the column is equilibrated with the given solute concentration and then stripped with a suitable eluent to remove the adsorbed solute from the surface and the eluent fraction is collected. The fraction is analyzed separately to estimate the amount of solute adsorbed to the surface. The measurement of alkylammonium ions in the fractions generally employ a time-consuming evaporation process and/or an expensive derivatization reaction where alkylammonium ions are measured photometrically as picrate complex or through gas chromatography (GC) with packed columns [22,29,30].

In contrast to LC, well established GC methods exist for the analysis of alkylamines in different media using modern capillary columns [31–33]. However, due to the highly polar and reactive nature of primary, secondary and lower aliphatic tertiary amines, their performance on the routine capillary GC columns is unsatisfactory. They interact with the free silanol group of the fused silica columns resulting in peak tailing, peak splitting or tend to adsorb and decompose on GC liners and columns, resulting in ghost peaks [34–36]. This tendency to adsorb and decompose is lower for tertiary amines as compared to primary and secondary amines, but peak tailing is still an obvious concern for smaller tertiary aliphatic amines like TEA [34,36,37]. Therefore, the selection of the right column: nature of the stationary phase (polarity), film thickness and inertness are critical factors for alkylamines analysis through GC [37,38].

The objective of this study was to develop an accurate method for acquiring the adsorption isotherm of TEA and TBuA (as alkylammonium ions) in eluents classically used in oligonucleotide separation in IP-RP chromatography. To do so we first needed to develop and validate an analytical method that could accurately determine the IPR content in the eluent. Our developed method will be simple and fast, which would neither require time consuming and expensive derivatization reaction nor specialized detector, to determine the amount of TEA and TBuA in a typical IP-RP chromatographic mobile phase i.e., acetonitrile-water. Secondly, we will develop a batch method for the acquisition of adsorption isotherm for tributylammonium acetate (TBuAA) and triethylammonium acetate (TEAAA). The focus of this part will be to reduce the amount of eluent required to strip off the adsorbed solute from the column. Finally, the developed analytical method will be used to measure the amount of IPR in the stripping eluent and acquire the adsorption isotherm for TEA and TBuAA. The aim of this study was not to do a systematic study of the adsorption of TBuAA and TEA under different conditions but to develop a method for the acquisition of adsorption isotherms of these compounds and then evaluate the nature of their adsorption isotherms as they have not been reported previously.

2. Theory

In this study, the adsorption isotherm was acquired using a batch method, in which the column was first equilibrated with an eluent containing the IPR at the feed concentration C₀. At equilibrium, the concentration of the IPR in the mobile phase (Cₑ) was the same as C₀, and the adsorbed amount of solute on the stationary phase was Qₑ. Thereafter, the IPR in the column was stripped by switching to an eluent without any IPR. The effluent was collected during the stripping phase and the amount of IPR in the collected fraction was analyzed. Using mass balance, the amount of IPR adsorbed to the stationary phase was calculated. This was done by recognising that the amount collected during the stripping phase is equal to the sum of the amount adsorbed to the stationary phase and the amount of IPR occupying the system dead
volume \((C_0V_{eq})\). To calculate the amount adsorbed, the amount of IPR in the dead volume of the system had to be subtracted from the amount measured in the stripping solution \((C_1V_1)\) as shown in Eq. (1):

\[
Q_{eq} = C_1V_1 - C_0V_{sys}.
\] (1)

In Eq. (1), \(C_1\) is the concentration of the collected effluent, \(V_1\) is the volume of the collected effluent and \(V_{sys}\) is the system’s dead volume which includes the column’s dead volume. The concentration of the IPR adsorbed to the stationary phase \((q_{eq})\) was calculated using Eq. (2):

\[
q_{eq} = \frac{Q_{eq}}{V_s}.
\] (2)

In Eq. (2), \(V_s\) is the volume of the stationary phase which is the sum of the volume of the silica particles and that of the coating \((\text{C18 ligand})\) attached to them. This was experimentally estimated by subtracting the determined dead volume of the column (Refer Section 3.6) from its geometric volume.

Error bars, in all the figures, are graphical representations of the confidence interval with a confidence level of 95\%. In Figs. (1–4), the standard uncertainty in the concentration was estimated using propagation of errors for the model fit to the linear standard curve \((y(x) = mx + b)\). Assuming that the errors in the concentration depicted on the x-axis for the standard curve, are negligible, the standard uncertainty in the measured concentration \((s_x)\) can be expressed as shown in Eq. (3) \([39]\):

\[
s_x = \frac{s_y}{m} \sqrt{1 + \frac{1}{b^2} + \frac{(\bar{y} - \bar{y})^2}{m^2SXX}}.
\] (3)

In Eq. (3), \(n\) is the number of points in the standard curve, \(k\) is the number of replicate measurements of the sample, \(\bar{y}\) is the average response from the replicate measurements of the sample and, \(SXX\) is the sum of the squares of the difference between each \(x\) and the mean \(x\) value. \(s_y\) is the standard error of the \(y\) values, which can be calculated as given by Eq. (4):

\[
s_y = \left(\frac{1}{n} \sum_{i=1}^{n} (y_i - (mx_i + b))^2 / (n - 2)\right)^{1/2}.
\] (4)

The confidence interval was estimated using Eq. (5):

\[
\bar{y} \pm t_{n-2} s_y / \sqrt{k}.
\] (5)

In Eq. (5), \(t\) is the Student’s \(t\)-value with \(n - 2\) degrees of freedom. To estimate the error in \(q_{eq}\) in Figs. (5 and 6), the error in the concentration of the collected fraction was first estimated as above, and then multiplied with the ratio between the volume of the collected fraction and the volume of the stationary phase.

3. Materials and methods

3.1. Chemicals and reagents

GC-grade n-pentane and LC-grade hexane were purchased from Merck (Darmstadt, Germany), while HPLC-grade acetonitrile (MeCN) and heptane were supplied by VWR international (Rue Carnot, Fontenay-sous-Bois, France). Triethylamine (TEA) and tributylamine (TBuA) - both \(> 99.5\%,\) tripripropylamine (TPrA) \(> 98.5\%,\) glacial acetic acid \(> 99.7\%,\) AR-grade sodium hydroxide (NaOH), uracil and sodium chloride (NaCl) \(> 99\%,\) were sourced from Sigma Aldrich (St. Louis, MO, USA). Milli-Q water purification system (Merck Millipore, Darmstadt, Germany) having a resistivity of 18.2 M\(\Omega\)-cm was used for solvents and standards preparation.

3.2. Instruments

The analyses of TEA and TBuA were performed using HP 6890 gas chromatograph attached to a flame ionization detector (Hewlett Packard, Germany). The column used was J&W DB-624, 30 m x 0.53 mm i.d. x 3 \(\mu\)m (Agilent Technologies, Santa Clara, CA, USA). A 10 \(\mu\)l syringe was used for the injection of samples.

The column used to determine the adsorption isotherm was a reversed phase XBridge BEH C18 Column, 4.6 mm x 150 mm, 3.5 \(\mu\)m from Waters Corporation (Milford, MA, USA). This column is packed with ethyl-bridged hybrid silica particles where the particles are synthesized by a co-condensation of tetraethoxysilane and bis[triethoxysilyl]ethane monomers \([40]\). As gathered from the manufacturer, the surface area of the column was 186 m\(^2\)/g, the average pore diameter was 140 Å and the total carbon content was 18.07\% with a surface concentration of 3.42 \(\mu\)mol/m\(^2\).

An Agilent 1260 Infinity II LC system (Waldron, Germany) equipped with Auto-sampler (G7129A), binary pump (G7112B), and detector (G7117C) was used for the measurement of the void volume/dead volume of the column. A separate PU-1580 Intelligent HPLC Pump (JASCO, Tokyo, Japan) was used for the adsorption and desorption process. Hettich centrifuge EBA 20, (Hettich GmbH & Co. KG, Tuttingen, Germany) was used for centrifugation.

3.3. Development of analytical method for the determination of TBuA and TEA

Separate stock solutions (10 mM) of triethylammonium acetate (TEAA), tributylammonium acetate (TBuAA) and tripropylammonium acetate (TPrAA) each containing 10 mM NaCl were prepared, by mixing equal amount (mole) of corresponding amine, acetic acid and NaCl in 50 % MeCN (MeCN water). 1.0 M NaOH solution was prepared in Milli-Q water. The standard solutions for the standard curve of TEAA and TBuAA were prepared from the stock solution by dilution with 50 \% MeCN. A known quantity of TPrAA as an internal standard was added into each calibration standard from the TPrAA stock solution. Subsequently, 1 mL of each standard was taken in a 15 mL glass tube, and 1 mL of 1.0 M NaOH was added into each one of them. This was followed by the addition of 1 mL n-pentane into each standard, before the glass tubes were tightly closed with lids. The tubes were then vigorously shaken for about 5 min, and then centrifuged at 4000 rpm (approximately 1700 \(g\)) for 5 min. After centrifugation, they were refrigerated at 4°C so as to avoid excessive evaporation of n-pentane. These standards were then, piecemeal, brought to room temperature, and the top layer of n-pentane was injected immediately into the GC. The same procedure was applied to all other standards and samples.

For the analysis, the gas chromatographic conditions were as follows. The initial oven temperature was maintained at 40°C without initial hold time, and it was ramped up at a rate of 40°C min\(^{-1}\) to 220°C, at which temperature, it was held for 3 min. Helium, at a flow rate of 5 mL min\(^{-1}\), was used as a carrier gas. The injector and detector temperatures were 230°C and 250°C, respectively. For the detector, the flow rates of hydrogen, nitrogen and air, were 40, 20 and 350 mL min\(^{-1}\), respectively. The analyses were performed in split mode, the split ratio being 1:2, and the injection volume was 1 \(\mu\)L.

3.4. Validation of the analytical method developed

The method was validated according to the International Council for Harmonization (ICH) guidelines for parameters like linearity, range, intra-, and inter-day precision and accuracy, the lower limit of detection (LOD) and the lower limit of quantitation (LOQ) \([41]\). The concentration of calibration standards for generating the calibration curve of TBuAA were 0.010, 0.050, 0.100, 0.200, 0.400,
0.600 mM while for TEtAA the calibration standards were 0.050, 0.100, 0.200, 0.400, 0.800, 1.600 mM. TPtAA, as an internal standard, at a concentration of 0.300 mM, was added into each calibration standard. The LOD and the LOQ were estimated at signal-to-noise ratios of 3:1 and 10:1 respectively, by gradually injecting decreasing concentrations of both the solutes. The noise was estimated as the difference between the highest and lowest points of the baseline around the retention time of the solute, and the signal was taken as the height of the peak from the baseline’s centre to the peak’s apex.

Intra-day precision and accuracy were determined by analyzing quality control (QC) samples at three different levels of concentration, five times each, in a single day. Similarly, inter-day precision and accuracy were determined by running the QC samples five times each on three successive days. Precision was reported as relative standard deviation (%RSD) and accuracy as the relative difference (% error or % bias) between the measured mean and the actual QC values. For TEtAA, the QC samples in concentrations of 0.10 mM, 0.50 mM and 1.50 mM were prepared in 50 v% MeCN from a different stock solution than the one used for the calibration standards. For TBUAA, QC samples in concentrations of 0.05 mM, 0.10 mM and 0.50 mM were prepared in 50 v% MeCN from a separate stock solution. Similarly, QC samples in 30 v% MeCN and in 70 v% MeCN were also prepared at the same concentration levels for both TBUAA and TEtAA to check the performance of the method, for varying compositions of acetonitrile-water.

3.5. Adsorption isotherm acquisition method

The XBridge BEH C18 column was equilibrated with 40 mM TBUAA solution prepared in 70 v% MeCN for about 45 min at a flow rate of 1 mL min\(^{-1}\) (adsorption). This column was then stripped with 70 v% MeCN containing 40 mM NaCl and the eluent was collected in fractions of 2.5 mL (desorption). Since the volume of the column was about 2.50 mL, the different fractions were named as column volume 1 (CV1), CV2, CV3 and so on. These fractions were extracted with n-pentane, and analyzed on GC using the procedure described in Section 3.3. Thus, the volume of the eluent required for the complete elution of the adsorbed TBUAA on the column was determined. The procedure was repeated for TEtAA, with the only difference being that the solution for adsorption was prepared in 30 v% MeCN, and stripped with 30 v% MeCN containing 40 mM NaCl.

3.6. Scheme for the acquisition of adsorption isotherm

For the acquisition of the adsorption isotherm for TBUAA, the column was equilibrated with 40 mM TBUAA in 70 v% MeCN (adsorption solution). It was then stripped with 70 v% MeCN containing 40 mM NaCl, and 75 mL of the eluent fraction called stripping volume or elution volume (30 CV) was collected. In order to calculate the concentration of TBUAA in the 75 mL fraction, a known volume of this fraction was diluted so as to bring it into the concentration range of the calibration curve. Then 1 mL of this diluted solution was extracted, and analyzed, as described in Section 3.3, to calculate the concentration of TBUAA.

This process was then repeated for other concentrations of TBUAA to acquire the adsorption isotherm (Refer Table 1). NaCl was added to keep the ionic strength constant in the adsorption solutions, as this would affect the surface potential. The process adopted for TBUAA was repeated for TEtAA (the difference being the same as the one referred to in Section 3.5).

The quantities of TBUAA and TEtAA adsorbed to the stationary phase were calculated using Eq. (1); \(V_{sys}\) in Eq. (1) is the sum of the void volume of the column and that of the tubes connected to the column used for adsorption and desorption process (Refer Section 3.5). Void volume (\(V_0\)) of the column was measured by connecting the column to the HPLC system. For experiments with TBUAA, 5\(\mu\)L of 0.1 mM uracil solution was injected at a flow rate of 1 mL min\(^{-1}\) in 70 v% MeCN as the mobile phase. The void volume (\(V_0 = 1.33 \text{ mL}\) of the column for TBUAA was calculated from the flow rate and retention time (\(t_0\)) of uracil. The void volume (1.30 mL) for TEtAA experiments was measured in 30 v% MeCN at the same flow rate and injection volume. Both void volumes were corrected for the void volume of the HPLC system. The total column volume was 2.49 mL, calculated geometrically using data provided by the vendor.

### Table 1

<table>
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4. Results and discussion

4.1. Development of analytical method for the determination of TBUA and TEtA

Under the given set of conditions (Refer Section 3.3), the alkylamines were present in the form of ammonium ions in a medium containing water, acetonitrile and acetic acid. A strong 1M NaOH solution was used to convert the ammonium ions into free amines, which were subsequently extracted into an organic solvent immiscible with the solvents in the medium referred to above, for injection into the GC. Hepatane has been regarded as the best option by Hartvig and Vessman for amine extraction from alkaline aqueous samples [42]. However, while analyzing the extract using GC, it was observed that TEtA was co-eluting with heptane. This made us abandon heptane and experiment with hexane instead. TEtA was either eluting closely with hexane, or observed to be producing peak tailing under different GC conditions. Hexane thereby was substituted by pentane, which turned out to be suitable. With pentane, the TEtA was well-separated from the solvent peak, and did not produce any peak tailing at the required set of chromatographic conditions (refer Fig. S1 in the supplementary material).

The calibration standards were prepared in the same matrix as the samples, and extracted with pentane in the same way as the actual samples. This is because pentane is very light (less dense) and volatile and extra care was needed to pipette pentane to achieve the required level of linearity and repeatability. As FID is relatively less sensitive to amines, splitless mode of injection was used to achieve a better sensitivity. But TEtA produced peak tailing in this mode, and therefore the lowest possible split ratio (1:2) for a 0.53 mm ID column, was the best compromise, to obtain a reasonably-good response in FID. Lowering the split ratio from 1:5 to 1:2 did not affect the peak shape, but improved the sensitivity. As TEtA is a relatively active compound and tends to deposit over the column, the oven temperature was maintained at 220°C for 3 min, to thwart the deposition.

As described in the Introduction (Section 1), TEtA produces peak tailing when analyzed with a standard 0.25 mm ID capillary column [34,35]. Therefore, it was decided that a wide-bore capil-
lary column covered with a thick layer of the stationary phase (3 μm) which has a limited residual silanol activity, should be used instead. Gopakrishnan and Devi, Raghuram and colleagues; and, Tian and fellow researchers [32,37,38] have used wide-bore capillary column GC method for the determination of dimethylamine and TEtA in drug substances and active pharmaceutical ingredients. However, they have either used headspace for sample injection and/or their matrix was non-aqueous. Headspace is not suitable for TBuA as it is not volatile (boiling point 214°C). Further, the method requires a longer equilibration time, which adds to the cost. It is also not present in routine GC laboratories and demands extra care and expertise. Similarly, Moore and colleagues [36] used GC capillary method for the measurement of TEtA in the veterinary medicine sarafluoxin hydrochloride. However, they had to go through the difficult process of treating the glass liner and glass wool with methanol potassium hydroxide to avoid peak tailing. As referred to earlier in this paper, due to the fact that TBuA and TEtA lack chromophore and produce severe peak tailing, their determination through liquid chromatography requires either a time-consuming derivatization reaction and/or needs a specialized detector [26,43].

The analytical method developed in this study obviates these requirements, and is especially effective in an aqueous-organic matrix, while being independent of the specialized sample injection or detection mechanism.

4.2. Validation of analytical method

Our proposed method showed good linearity for both TBuA and TEtAA. The response of TBuA was linear from 0.010 to 0.600 mM with a coefficient of determination R² of 0.9997. TEtAA showed linear response from 0.050 to 1.600 mM, with R² of 0.9990. (Refer to Figs. S2 and S3 in supplementary information, in which the slopes, intercepts and calibration curves for TBuA and TEtAA have been presented). The linearity for the TBuA and TEtAA calibrations was further confirmed by refitting the calibration data back to the calibration models. The back-calculated concentration for each calibration standard in both the curves deviated by less than 15% from the respective true concentration (Refer to Tables S1 and S2 in supplementary information). LOD and LOQ for TBuA were 0.005 mM and 0.010 mM respectively, while the corresponding values for TEtAA were 0.010 mM and 0.050 mM respectively.

The intra-day and inter-day precision and accuracy values have been summarized in Table 2. The relative standard deviation (RSD) values for intra-day precision and inter-day precision, for TBuA ranged from 1.60% to 2.61% and 1.21% to 2.91%, respectively. The corresponding ranges for TEtAA were 1.07% to 2.77% and 2.13% to 3.76% respectively. The relative error or percentage-bias for intra-day and inter-day accuracy of TBuA ranged from -1.40% to 1.52% and -1.36% to 2.37% respectively. The corresponding ranges for TEtAA were -0.55% to 3.08% and -1.55% to 0.99% respectively. The data used for calculation of intra and inter day precision and accuracy have been reported in Tables S3 and S4 in supplementary information. Based on the precision, accuracy and linearity data, the ranges of this analytical method for TBuAA and TEtAA are 0.050-0.500 mM and 0.100-1.500 mM, respectively.

While the calibration and quality control standards used for validation were prepared in 50 % MeCN, we decided to test the method with quality control standards prepared in 30 % MeCN and 70 % MeCN, as described in Section 3.3. The precision and accuracy of these results have been tabulated in Table 3. All these results indicate that our method is suitable for the determination of TBtAA and TEtAA over the range of 30 % to 70 % MeCN. The data used for the calculations of precision and accuracy in 30 % and 70 % MeCN have reported in Tables S5 and S6 respectively in the supplementary materials. This method is also applicable to different methanol-water compositions, but validation of the method for that media is not within the scope of this paper. It will be useful particularly for the determination of TBuAA and TEtAA in typical chromatographic mobile phases in gradient elution, and potentially for the measurement of residual TBuA and TEtA in oligonucleotide drugs.

4.3. Adsorption isotherm acquisition method

The adsorption isotherm was investigated by first equilibrating the column with eluent containing IPR. Thereafter, all the adsorbed IPR was eluted by using an eluent not containing any IPR. The stripping solution was collected and the amount of IPR in it was determined. To improve the accuracy of the adsorption data, and simplify the acquisition method, all the adsorbed IPR must be stripped, while minimising the volume of the solution.

To investigate how much stripping solution is required, the XBridge BEH C18 column was equilibrated with 40 mM TEtAA prepared in 30 % MeCN for 45 min. This was then stripped with 30 % MeCN and the fractions were collected (as described in Section 3.5), and analyzed (as in Section 3.3), to determine the amount of TEtAA in each of them. Fig. 1 shows the plot of the concentration of each fraction against the column volume (CV) of stripping solution used. As can be gathered from Fig. 1a, at 10 CV, the amount of TEtAA eluted from the column was below the LOQ value. However, TEtAA continued to elute at LOD until the 25th CV, at which point, it was completely stripped off the column. Fig. 1b highlights the latter part of Fig. 1a, where the concentration of TEtAA in the stripping solution decreases gradually.

TBuA has a solubility limit, specifically at lower concentrations of acetonitrile in the eluent. As 30 % MeCN was used as an eluent for the adsorption process; it was decided to limit the TBuA concentration to a maximum of 40 mM, to obviate any solubility problem. The concentration of TEtA was maintained on par with TBuA, to enable comparison between the adsorption of the two solutes.

Table 2:
The intra-day and inter-day precision and accuracy studies of TBuAA and TEtAA in 50 % MeCN.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration (μM)</th>
<th>Measured Concentration [μM]</th>
<th>Precision (%)</th>
<th>Accuracy%</th>
<th>Measured Concentration [μM]</th>
<th>Precision (%)</th>
<th>Accuracy%</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBuAA</td>
<td>0.050</td>
<td>0.0508±0.0013</td>
<td>2.61</td>
<td>1.52</td>
<td>0.0512±0.0015</td>
<td>2.91</td>
<td>2.37</td>
</tr>
<tr>
<td></td>
<td>0.100</td>
<td>0.0986±0.0018</td>
<td>1.82</td>
<td>-1.40</td>
<td>0.0986±0.0020</td>
<td>2.04</td>
<td>-1.36</td>
</tr>
<tr>
<td></td>
<td>0.500</td>
<td>0.4950±0.0079</td>
<td>1.60</td>
<td>-1.00</td>
<td>0.4965±0.0061</td>
<td>1.21</td>
<td>-0.71</td>
</tr>
<tr>
<td></td>
<td>1.000</td>
<td>0.1013±0.0011</td>
<td>1.07</td>
<td>1.07</td>
<td>0.1010±0.0037</td>
<td>3.76</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>1.500</td>
<td>0.5054±0.0140</td>
<td>2.77</td>
<td>1.07</td>
<td>0.4923±0.0112</td>
<td>2.27</td>
<td>-1.55</td>
</tr>
<tr>
<td></td>
<td>2.000</td>
<td>1.4917±0.0253</td>
<td>1.69</td>
<td>-0.55</td>
<td>1.4908±0.0217</td>
<td>2.13</td>
<td>-0.22</td>
</tr>
</tbody>
</table>

Note: The measured concentrations are expressed as mean ± standard deviation, n=5.
Table 3
Precision and accuracy studies of TBuAA and TEtAA in different compositions of acetonitrile-water.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Reference Concentration (QCs) [mM]</th>
<th>30 v% MeCN</th>
<th>70 v% MeCN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[mM]</td>
<td>Precision (RSD,%)</td>
<td>Accuracy (Bias,%)</td>
</tr>
<tr>
<td>TBuAA</td>
<td>0.050</td>
<td>0.0511±0.0009</td>
<td>1.78</td>
</tr>
<tr>
<td></td>
<td>0.100</td>
<td>0.1028±0.0008</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>0.500</td>
<td>0.4992±0.0074</td>
<td>1.49</td>
</tr>
<tr>
<td>TEtAA</td>
<td>0.100</td>
<td>0.1024±0.0019</td>
<td>1.85</td>
</tr>
<tr>
<td></td>
<td>0.500</td>
<td>0.5119±0.0060</td>
<td>1.18</td>
</tr>
<tr>
<td></td>
<td>1.500</td>
<td>1.4971±0.0051</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Note: The measured concentrations are expressed as mean ± standard deviation, n = 3.

The same experiment was conducted for 40 mM TBuAA in 30 v% MeCN. In this case, more than 180 CV (greater than 450 ml) were needed to completely elute TBuAA (below LOD) from the column surface. This indicates a very strong hydrophobic interaction of TBuAA with the nonpolar stationary phase of the column, which can be explained by the very high log P value of tributylamine (4.46), vis-à-vis TEtAA (1.45) [44]. The elution strength of the mobile phase (30 v% MeCN) was not sufficient to elute the adsorbed TBuAA from the column. Consequently, it was impractical to use 30 v% MeCN as the stripping eluent as that would render it impossible to measure the lower points in the adsorption isotherm for TBuAA. Besides, it was time-consuming and required a large volume of organic solvent.

As the hydrophobicity of TBuAA is greater than that of TEtAA, less of the former is stripped, as compared to the latter. Increasing the amount of MeCN in the stripping solution could improve the stripping efficiency. To test it, 40 mM TBuAA was prepared in 70 v% MeCN. The column was equilibrated with it and then eluted with 70 v% MeCN. The desorption curve depicting the results of the analysis of the collected fractions is depicted in Fig. 2. It indicates that at 18 CV, the concentration of TBuAA dropped below the LOQ value, but it kept eluting at the LOD until 50 CV, at which point,

Fig. 1. Elution of 40 mM TEtAA adsorbed on the column using 30 v% MeCN. C is the concentration in different fractions of the stripping solution. (a) Complete elution until TEtAA amount becomes lower than the LOQ and (b) the last part of the elution profile.

Fig. 2. Elution of 40 mM TBuAA adsorbed on the column using 70 v% MeCN. C is the concentration in different fractions of the stripping solution. (a) Complete elution profile until TBuAA amount becomes lower than the LOQ, (b) the initial part of elution profile and (c) the last part of the elution profile.
it was completely eluted from the column. Although the desorption of TBuAA from the column increased considerably, the total volume of eluent required for complete elution of TBuAA was still quite high – 125 mL, making it difficult to measure the lower points in the adsorption isotherm of TBuAA.

In order to further improve the elution of TBuAA from the surface of the stationary phase, two alternatives were considered. The first one was to further increase the MeCN content of the stripping eluent. However, one could speculate that as the nature of the adsorption to the stationary phase is both electrostatic and solvophobic, this alternative would strengthen the electrostatic interaction. The second option was to increase the ionic strength of the eluent by adding some neutral salt to the stripping solvent. From a fundamental perspective, this will decrease the electrostatic interaction with the stationary phase, thereby reducing the degree of adsorption. From earlier studies, it is known that the adsorption of a charged amine solute can be described using a bi-Langmuir adsorption isotherm [45], which is simply a linear combination of two Langmuir adsorption models with two different adsorption sites. Samuelsson and colleagues [45] showed that for metoprolol on the XBridge column, predominantly solvophobic interactions resulted in sites with low adsorption strength and high capacity; while more electrostatic interactions with the charged silanols in the stationary phase, could explain the presence of sites with high adsorption strength and low capacity. In that study, the strong adsorption sites had around 20% of the total saturation capacity of the column.

Inspection of the desorption curve in Fig. 2, reveals that TBuAA eluted very fast to start with, and at 4 CV, about 90% of it had been stripped from the column. Thereafter, the rate of stripping slowed down considerably. It can be inferred that the weakly-adsorbed TBuAA eluted early, while electrostatic interaction existing between it and the column’s surface resulted in the extreme tailing which was observed.

Fig. 3 shows the desorption curves for TBuAA using 70% MeCN containing 40 mM NaCl as the stripping solution. This uncovers the presence of electrostatic interaction as sodium ions would compete with tributylammonium ions for the negative sites on the column surface. In this case (with addition of NaCl), the LOQ was reached earlier at 12 CV, compared to 18 CV without NaCl. TBuAA was completely eluted from the column at 30 CV, vis-à-vis 50 CV without the addition of NaCl. Therefore, it was decided to use 30 CV - 75 mL of 70% MeCN containing 40 mM NaCl - to determine the different points in the adsorption isotherm of TBuAA.

NaCl was also added to the eluent to desorb TEtAA from the column while keeping the elution strength (amount of % MeCN) constant (see Fig. 1). The column was equilibrated with 40 mM TEtAA prepared in 30% MeCN and then stripped with 30% MeCN containing 40 mM NaCl (as described before), and the resulting desorption curve has been shown in Fig. 4. It shows that the amount of TEtAA eluting from the column became lower than the LOQ at 6 CV, and LOD was reached already at 10 CV, which made us decide to use 10 CV (25mL) to determine the different points in the adsorption isotherm of TEtAA. Ståhlberg [22] used more than 80 CV to strip off the adsorbed tetrabutylammonium ions from C18 LiChrosorb column, using 80% acetonitrile-water as the stripping solvent while Hung and Taylor [21] used more than 200 CV to fulfil the same function, from ODS Hypersil column using neat ethanol as a stripping solvent. Although the solutes used by Ståhlberg [21] and Hung and Taylor [22], were more hydrophobic than the ones used in this study, the quantity of eluent required for stripping was very large. Additionally, they have not commented on the sufficiency of the quantity of eluent used by them. While their methods also involved the evaporation of the fractions collected, and the conversion of the alkylammonium ions into a picate complex, the method developed in this paper requires lesser quantities of solvents, and does not include the time-consuming evaporation and complexation processes.

The addition of NaCl to the stripping eluent drastically improved the desorption of both TEtAA and TBuAA from the column. For TEtAA, the stripping eluent volume decreased from 25 CV to 10 CV. In the case of TBuAA, increasing the concentration of MeCN from 30% to 70% resulted in a decrease in the requirement of stripping eluent from 180 CV to 50 CV. When NaCl was added, a further decrease down to 30 CV was registered; an overall improvement of 6 times.

The batch method developed in this study is slower than the dynamic HPLC method (which is beyond the scope of this study), but quicker and more accurate than the existent batch methods. If properly planned, the adsorption isotherm for TEtAA (7 data points) can be acquired in 8-9 h and the adsorption isotherm for TBuA can be acquired in 15-16 h.

4.4. Adsorption isotherms

Fig. 5 presents the adsorption isotherm of TBuAA using 70% MeCN as the eluent. The adsorption data for TBuAA was fitted to the three simple models: linear, Langmuir and anti-Langmuir model (Fig. S4 in supplementary information). These model fits were also statistically evaluated, by calculating the R²- and adjusted R²-values (Table S6 in supplementary information). From the model fit and statistical evaluation, it is clear that the adsorption data for TBuAA fits best to anti-Langmuir model (type III adsorption isotherm). In this case, the amount of TBuAA adsorbed to the column surface increases slowly at lower concentrations, and then rises rapidly at higher concentrations. This could indicate the formation of multiple layers.

Mostly, type I adsorption isotherms have been reported for tetrabutylammonium ions by several researchers using different chromatographic conditions [21-25]. All these observations differ from the adsorption isotherms obtained for TBuAA, in this particular study. These differences were confirmed by repeating the experiments. This may indicate that the behavior of tetrabutylammonium ion which is a permanently-charged species is different from tributylammonium ion, the charge of which depends on the pH of the medium under investigation.

Trialkylammonium acetate, which was used as a buffer in this study, has a low buffering capacity. Subirats and fellow researchers
Fig. 4. Elution of 40 mM TEtAA adsorbed on the column using 30 v% MeCN containing 40 mM NaCl. C is the concentration in different fractions of the stripping solution. (a) Complete elution profile until TEtAA amount becomes lower than the LOQ and (b) the last part of elution profile.

Fig. 5. Adsorption isotherm for TBuA on reversed phase XBridge C18 column in 70 v% MeCN. C_{eq} is the equilibrium concentration of TBuA in solution and q_{eq} is the equilibrium concentration of TBuA in the stationary phase.

Fig. 6. Adsorption isotherm for TEtAA on reversed phase XBridge C18 column in 30 v% MeCN. C_{eq} is the equilibrium concentration of TEtAA in solution and q_{eq} is the equilibrium concentration of TEtAA in the stationary phase.

[46] have studied the effect of the composition of the organic solvent on the pH of the mobile phase buffers and the pK_{a} values of the solute. By extrapolating from their results, the pH of 70 v% MeCN containing equal amounts of acetic acid and TBuA is 8.37, and the pK_{a} value of TBuA in 70 v% MeCN is 9.95. This means that in 70 v% MeCN, about 97% of TBuA would remain in charged form. The charged and uncharged forms have different affinities to the stationary phase. This could have resulted in the deviation of adsorption isotherm from a typical type I to less-common type III adsorption isotherm for tributylammonium ion under the investigated conditions. Researchers in the past [45,47–49] have observed that the adsorption isotherm for basic compounds deviate from type I close to the pK_{a} of the solute for different commercial C18 phases [45,47–49].

Many models currently used for the retention of charged analytes in IP-RP chromatography- for example, Cecchi’s model [16] – assume type I adsorption isotherm for the IPRs. As discussed earlier, most of those reported adsorption isotherms for tetraalkylammonium ions were also type I. However, in this study, type III adsorption isotherm was obtained for TBuA. This implies that the same model needs to be modified for oligonucleotides separations using TBuA as IPR. How this will affect the selectivity of oligonucleotides and related impurity needs to be investigated in a separate study.

The adsorption isotherm for TEtAA in 30 v% MeCN is presented in Fig. 6. From the model fit and statistical evaluation (Fig. S5 and Table S6 in supplementary information), it is evident that the TEtAA system requires a Langmuir model (type I adsorption
isotherm) to describe the observed adsorption data, which cannot be described by taking recourse to a linear model. Estimating the charged and uncharged form in 30 v/v MeCN using a similar method as above, 99.7% TEtAA will be present in charged form. In this case the adsorption isotherm of TEtAA is a single component system and that may be the reason that we obtained a typical type I adsorption isotherm under the investigated conditions.

In order to confirm that the time required for the attainment of equilibrium of the column is sufficient, the breakthrough volumes for the lowest concentrations of TBuA and TEtAA were estimated from their respective adsorption data [18]. The estimated breakthrough volume is less than 3 min for the TBuA system and close to 2 min in the TEtAA system. This indicates that 45 min should be more than sufficient to equilibrate the column at all concentration levels used for the acquisition of adsorption isotherms.

5. Conclusion

Determination of alkylamines by traditional liquid chromatography is very difficult due to their highly polar nature, presence in charged form in real samples and absence of suitable chrophore. Therefore, they either require a derivatization reaction which takes a long time to be completed, or they need a specialized column and detector. In this study, a very simple method was developed for the determination of alkylamines in typical reversed-phase chromatographic mobile phase (acetontitrile-water). The alkylamines, which were present in charged form in the mobile phase, were first neutralised by treatment with a strong alkaline solution. This solution was then extracted with n-pentane and analyzed on GC. A special GC column coated with a thick film of stationary phase was used for the analysis. The developed method showed good linearity, accuracy and precision.

This analytical method was then used for the development of a method for the acquisition of adsorption isotherms of TBuAA and TEtAA. TBuAA, by virtue of its greater hydrophobicity with respect to TEtAA, interacts more strongly with the stationary phase as compared to TEtAA. However, this interaction decreases as the acetontitrile content in the eluent increases. The adsorption isotherms were acquired by equilibrating the reversed phase C18 column with different concentrations of IPRs, and then eluting with solvent until all the IPRs adsorbed on the column, are stripped off the stationary phase. The stripping eluent was collected and analyzed. TBuAA at 70 v/v MeCN in the investigated concentration interval showed a type III adsorption isotherm, while for TEtAA at 30 v/v MeCN, it was type I. In future studies, this method can and will be used to assess the effects of organic modifier, temperature and counter ions like HFIP on the adsorption of TBuAA and TEtAA on C18 phases. This knowledge is crucial to gain a deeper understanding of the separation mechanisms of oligonucleotides as discussed in the introduction of this paper. The analytical GC method can also be utilized to measure residual TBA and TBuA in oligonucleotide drugs. The GC method can be further improved by using a more nonpolar column having a thicker layer of stationary phase coated on the wall or a specialized column for amines. Hexane and heptane can also be adopted as extraction solvents. The sensitivity of this method can be improved if nitrogen-phosphorous detector or mass spectrometer is used instead of FID.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Abdul Haseeb: Conceptualization, Methodology, Software, Investigation, Validation, Formal analysis, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization. Maria Rova: Conceptualization, Methodology, Validation, Writing – review & editing, Supervision. Jörgen Samuelsson: Conceptualization, Methodology, Software, Investigation, Validation, Formal analysis, Data curation, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Data availability

All data is presented in the manuscript or the supplementary material.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2022.463857.

References

A. Hasouseb, M. Rova and J. Samuelsson


