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Reversed-Phase Ion-Pair Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry for Separation, Sequencing and Mapping of Sites of Base Modification of Isomeric Oligonucleotide Adducts using Monolithic Column

Vaneet K Sharma¹, James Glick¹, and Paul Vouros^{1,*}

¹Department of Chemistry and Chemical Biology, Barnett Institute, Northeastern University, Boston, MA 02115, USA

Abstract

In this manuscript, an efficient high resolution reversed phase - ion pairing- liquid chromatography electrospray ionization tandem mass spectrometry (RP-IP-LC-MS/MS) method for separation of isomeric modified oligonucleotides using a polymeric (styrene-divinylbenzene) monolithic capillary column is presented. The effects of different ion pairing reagents (IPR), their concentration, mobile phase additives and conditions were evaluated towards achieving the highest possible resolution and chromatographic separation of isomeric oligonucleotides. Ion pairing reagents and mobile phase conditions were evaluated using as model N-acetylaminofluorene [AAF] adducted ss- oligonucleotides (*CCC CGA GCA ATC TCA AT*). The optimized mobile phase conditions were then applied for the mapping of sites of base modification of AAF adducted 15 base pair oligonucleotide fragments containing codon 135 of the p53 gene and for profiling a complex synthetic oligonucleotide mixture. The optimized method utilizes a monolithic poly(styrene-divinylbenzene) capillary column, triethylammonium bicarbonate as ion pairing reagent and methanol as organic modifier to perform IP-RPLC-ESI-MS/MS separation. The results show that the method is simultaneously applicable not only to oligonucleotide fragments adducted separately by different carcinogens but also to the analysis of multiple adducts in the same oligonucleotide fragment in a single experiment. The method presents itself as a tool for the identification, characterization and mapping of oligonucleotide adducts as biomarkers for DNA damage from carcinogens.

Keywords

ion pairing reagent; liquid chromatography; mass spectrometry; Oligonucleotide; DNA-adducts; tandem mass spectrometry

1. Introduction

It has been extensively reported that carcinogen adducts, derived from exogenous sources form covalent bonds with DNA [1,2]. These DNA adducts may be differentially removed by

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*p.vouros@neu.edu, Phone: 617-373-2840, Fax: 617-373-2855.

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the human cellular machinery or otherwise repaired by various DNA repair mechanisms including nucleotide excision repair (NER). In the event that the DNA repair mechanisms fail to perform their task, it is speculated that this may induce mutations in the genes [3,4].

In the last decade liquid chromatography interfaced with mass spectrometry [LC-ESI-MS] has emerged as a central analytical technique for the characterization of nucleic acids but, in the case of DNA adducts, most of the reports are limited to their analysis as monomeric species [5,6,7,8,9]. Detection of DNA adducts as monomeric species is a reflection of the extent of exposure to carcinogens, providing quantitative analyses but fails to provide a more complete assessment of the risk associated with these adducts due to lack of knowledge about the adduct(s) location within the DNA sequence.

Presently, there are few [10, 11] techniques or approaches available which can probe the relationship between the sequence selectivity of carcinogen adducts and tumor mutations. There are well established techniques which when used separately can identify or quantify the DNA adducts or identify gene mutations but, as adducts associated with DNA are lost prior to sequencing, next generation DNA sequencing or PCR based techniques are not viable for assessing the role of DNA adducts. It is this inherent failure of the PCR based techniques to analyze oligonucleotide adducts that has presented opportunities to liquid chromatography mass spectrometry based approaches [12, 13].

A key advantage of LC-ESI-MS lies in its being the least labor-intensive technique along with its ability to identify, characterize and thus locate the exact position of the adduct on the oligonucleotide. The LC-ESI-MS/MS technique could be further made useful by performing high throughput chromatography using UPLC or by use of less expensive alternatives like polymeric monolithic stationary phase columns [14, 15]. The polymeric monolithic columns consist of a continuous “one piece” fritless, porous, highly crosslinked polymer bed comprising micropores and macropores with no interparticular voids which results in rapid mass transfer and thus higher flow rates [16, 17, 18].

Traditionally anion exchange chromatography has been the most widely used mode for analysis of oligonucleotides because of their negatively charged phosphate backbone [19, 20]. However ESI-MS is not compatible with mobile phases containing high concentration of nonvolatile salts or high strength buffers. Reversed-phase liquid chromatography is nowadays the most commonly used interface with ESI-MS but, as oligonucleotides are also difficult to retain on the nonpolar stationary phase of the reversed phase column, an ion pairing reagent (IPR) is added to the mobile phase to increase retention [21, 22, 23]. An ion pairing reagent possesses a dual functionality, i.e., a positively charged group, typically an amine, and a hydrophobic component in its structure. The use of IPR not only improves the retention of oligonucleotides onto the nonpolar stationary phase by making them neutral and hydrophobic in nature but also minimizes alkali cation adduction.

Previously, it has been reported that there are a number of chemicals which act as carcinogenic adducts and form covalent bonds with nucleobases over a wide range along the human DNA. This is evident in the case of the p53 tumor suppressor gene, which is mutated in more than 50 % of cancers and these different cancers might have originated from different exposures like environmental, dietary, UV radiations etc [24, 25]. Thus in the same gene there is potentially carcinogenic adduction taking place simultaneously at multiple sites with varying preferences. Adduction at multiple sites by a given carcinogen results in formation of isomeric oligonucleotide fragments and as a result, an appropriate volatile ion pairing reagent is needed to provide an efficient RP-LC-ESI-MS/MS separation of these isomeric species. There are only few reports regarding the use of LC-MS/MS for separation of positional isomers of carcinogenic adducted oligonucleotides [26, 27, 28, 29]. Harsch et

al in 2000 reported the LC-ESI-MS/MS identification of the adduct sites of an oligonucleotide derived from the HPRT gene sequence, following its reaction with benzo[c]-phenanthrene diol epoxide using PRP-1 reversed phase PS-DVB particle column (1.0X150mm, 3 μ m) and 20 mM ammonium acetate as an ion pairing reagent [27]. Although the above mentioned report successfully identified the sites of adduction, it either required large sample quantities or suffered from the buildup of higher back pressure over time which is inherent to a particle based column. Later, in 2007, Xiong et al, reported an ion pair reversed phase nano high performance liquid chromatography electrospray ionization mass spectrometry (IP-RP-nanoHPLC-ESI-MS) method using a monolithic poly(styrene-divinylbenzene) (PS-DVB) column to identify and separate a 14-mer isomeric ($\pm$)-anti-7r,8t-dihydroxy-9t,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene [($\pm$)-anti-BPDE] adducted oligonucleotide containing codon 157 of the p53 gene [28]. A related study involving codon 157 was also reported by Chowdhury *et al* in 2007 for analysis of a ($\pm$)-anti-BPDE or 4-aminobiphenyl (ABP) adducted double stranded oligonucleotide using an aquity UPLC BEH C18 column (1.7 μ m, 1.0 mm $\times$ 100 mm). Ammonium acetate (10 mM) was used as ion pairing reagent without however achieving the chromatographic separation of the isomeric oligonucleotide adducts [29].

In this manuscript, we discuss the fabrication and characterization of a monolithic PS-DVB capillary column, the evaluation of different ion pairing reagents, the effect of the mobile phase conditions on the chromatographic separation of positional isomers of oligonucleotide adducts, and further evaluation of this on-line PS-DVB monolith-IP-RP-LC-ESI-MS/MS mapping method for the separation and structural identification of a complex mixture comprised of unadducted, singly or multiple AAF adducted oligonucleotides.

2. Materials and methods

2.1 Chemicals and oligonucleotide samples

Caution: AAF is carcinogenic and should be handled with appropriate personal protective equipment.

Acetonitrile, methanol, and water (all HPLC grade) were obtained from Fisher Scientific (Pittsburgh, PA). Divinylbenzene (synthesis grade), styrene (synthesis grade), decanol (synthesis grade), tetrahydrofuran (THF) (analytical reagent grade), azobisisobutyronitrile, (synthesis grade) were purchased from Sigma-Aldrich (St. Louis, MO). The triethylammonium acetate (1.0M, pH 7), and triethylammonium bicarbonate (1.0M, pH 8.4–8.6) salts were purchased from Sigma-Aldrich (St. Louis, MO). Aqueous solution of bases such as triethylamine, tripropylamine, hexylamine, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), *N,N*-dimethylcyclohexylamine, *N,N*-dimethylbutylamine, purchased from Sigma-Aldrich (St. Louis, MO), were tested as their ammonium salts with 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) as buffering agent or as acetate or bicarbonate salt. A 1.0 M stock solution of *N,N*-dimethylcyclohexylammonium bicarbonate (CycHDMAB), hexylammonium bicarbonate or *N,N*-dimethylbutylammonium bicarbonate (DMBAB) was prepared by passing carbon dioxide through the corresponding amine solution at 5°C until pH 8.4–8.6 was reached [30]. A 1.0 M stock solution of *N,N*-dimethylcyclohexylammonium acetate (CycHDMAA) was prepared by adding acetic acid dropwise to the *N,N*-dimethylcyclohexyl amine until pH 8.4–8.6 was reached [31]. *N*-acetoxy-*N*-acetylaminofluorene [AAAF], was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Midwest Research Institute, Kansas City, MO). The synthetic oligodeoxynucleotides (17 mer, 18 mer oligonucleotide) were obtained from the Midland Certified Reagent Company. The 12 mer, 15 mer base pair oligonucleotides were obtained from Sigma® Life Science and Integrated DNA Technologies (Coralville, IA). The 18 nucleotide oligonucleotide used has an –OH group

missing from the base of 10th nucleotide (guanine) from 5' end. The oligonucleotides were dissolved in water without further purification and were used as stock solutions with a concentration of 1 µg/µL. The *in-vitro* AAF adducted oligonucleotide was prepared as described elsewhere and used directly for analysis without further purification [32, 33]. A typical yield for in-vitro AAF adduction was 40–50% depending upon the oligonucleotide sequence.

2.2 PS-DVB polymeric monolithic capillary column: Preparation and characterization

Our group has previously reported on the use of the monolithic PS–DVB column as compared to packed PS-DVB or C-18 reversed-phase columns to separate oligonucleotide adducts [28]. These monolithic (PS-DVB) capillary columns are fabricated by following a two-step procedure (i) Pre-treatment of the capillary inner wall and, (ii) polymerization of PS-DVB monolithic capillary column [18]. The quality of the modified wall is evaluated by its wetting properties using the capillary rise method, which is a measure of the contact angle, θ with a higher contact angle reflecting a better inner wall treatment. Only those silica capillaries which have $\theta > 85^\circ$ were used further for subsequent in-situ polymerization at 70°C for 16 hours by treating with a mixture of divinylbenzene, styrene, decanol, and THF at a volume ratio of 3.33:3.33:9.0:1.0 and 10mg/mL of α, α' -Azobisisobutyronitrile (AIBN).

The performance of the monolithic PS-DVB stationary phase is dependent on the ratio of monomers and porogens composition and a small change in that ratio during fabrication affects the pore diameter and stationary phase morphology. Therefore, before these columns can be used for any applications, they need to be characterized in order to define their properties. One of the most important properties to be determined in these monolithic PS-DVB capillary columns is their flow resistance which is characterized by the column permeability, K_F and is calculated using Darcy's equation [34, 35, 36]. For our monolithic PS-DVB column (0.25X95 mm, 6 µL/min flow rate) K_F was found to be $2.96 \times 10^{-14} \text{ m}^2$ and this higher value of specific permeability in turn is due to the high porosity (~82%) of the monolith column which means that only ~18% of the column is occupied by porous polymeric material. It is because of this higher porosity and corresponding lower flow resistance that use of higher flow rates results in lower back pressure in the PS-DVB monolithic columns. The average micropores size through which the analytes flow was determined to be 709 nm using the Kozeny-Carman equation (Table 1).

The monolithic PS-DVB capillary column fabricated and used in this study has shown excellent durability and retention-time repeatability for more than 1000 injections. High run to run repeatability showed, occurrence of minimal swelling or shrinking when this polymeric column was exposed to washing under harsh conditions such as 100% methanol, 100% acetonitrile or 100% isopropanol. To demonstrate the robustness of this monolithic PS-DVB column a retention-time repeatability study over 5 months for more than 1000 injections was performed and the retention time of a 17 mer oligonucleotide, CCT ACC CCT TCC TTG TA (5031.39 Da) was found to vary by an average of no more than of ± 30 seconds. The mobile phase were freshly prepared every day and no appreciable carryover was observed for the PS-DVB monolithic column, Thus, it could be inferred that the major advantages of using a monolithic PS-DVB column is virtually no contamination buildup, easy column regeneration and robust nature.

2.3 Reversed phase liquid chromatography electrospray ionization ion trap mass spectrometry (RP-IP-LC-ESI-IT-MS)

An Agilent series binary pump with degasser was used to generate solvent flow at a rate of 0.3 mL/min (Agilent Technologies, Palo Alto, CA). A microTee (Upchurch Scientific, Oak Harbor, WA) and polyimide coated fused-silica capillary tubing was used to split the flow

rate to 6 $\mu\text{L}/\text{min}$, resulting in a split flow ratio of 50/1. A six-port microbore valve (VICI, Valco, Houston, TX) equipped with an external sample loop of 1 μL was used for manual sample introduction. In general, mobile phase A consisted of a pure aqueous solution containing mM range concentration of ion pairing reagent, while mobile phase B consisted of 100% methanol. Mobile phases were prepared fresh everyday and degassed prior to use.

ESI-MS was performed on a LCQ Deca quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an electrospray ion source operated in the negative ionization mode. The instrument was tuned in the negative ion mode by infusion at 5 $\mu\text{L}/\text{min}$ of a 20 picomol/ μL solution of single-strand 15-mer oligonucleotide 5'-TGTTTTGCCAACTGG-3' in 50:50 (v/v) 25 mM TEAB/methanol using a syringe pump (Harvard Apparatus, Holliston, MA) equipped with a 250 μL glass syringe (Hamilton, Reno, NV). The PS-DVB monolithic capillary column was directly connected to the electrospray capillary (fused silica, 105 μm o.d., 40 μm i.d., Polymicro Technologies) by means of a microtight ZDV union (Upchurch Scientific, Oak Harbor, WA). For analysis the ESI-MS was operated in negative ion detection mode with an electrospray voltage of 5.5 kV and a nitrogen sheath gas flow of 15–20 arbitrary units (LCQ deca). The temperature of the heated capillary was set to 210 $^{\circ}\text{C}$. Total ion chromatograms and mass spectra were recorded on a personal computer using Xcalibur software version 1.4 (Thermo Finnigan, San Jose, CA). The LC-MS/MS experiments were conducted for the scan range m/z 700–2000 and data dependent MS/MS spectra were acquired at a collision width of 2 Da and relative collision energy of 30%.

3. RESULTS AND DISCUSSION

An environmental carcinogen has many options on where to form a covalent adduct on the oligonucleotide sequence and it is the probability of these interactions between the carcinogen and various nucleobases of an oligonucleotide which may yield positional isomers [37]. These resulting oligonucleotide adducts have the same molecular masses, similar oligonucleotide backbone and only differ in the location of adduct on the backbone. Thus they not only have to be efficiently separated but also sequenced to locate the exact position of adduction.

As the focus of this study was to develop a high resolution separation method for polyanionic isomeric oligonucleotide adducts, we first evaluated various volatile ion pairing reagents in terms of their effectiveness for the chromatographic analysis of the 17 nucleotide N-acetylaminofluorene [AAF] adducted oligonucleotide (CCC CGA GCA ATC TCA AT) (Figure 1). N-acetylaminofluorene [AAF] a potent bladder carcinogen which does not require metabolic activation and binds *in-vitro* preferentially to the guanine nucleobases to form N-acetyl-N-(guan-8-yl)-2 aminofluorene (C-8-AAF-dGuo) adducts was chosen as a model for this study [26, 38].

Once the optimum ion pairing reagent had been determined the effects of flow rates and linear gradients on chromatographic analysis were also investigated. Following optimization of the mobile phase conditions, this PS-DVB monolith-IP-RPLC-ESI-MS/MS method was used for direct detection and mapping of selected AAF adducted oligonucleotide fragments individually or in diverse mixtures.

3.1 Optimization of mobile phase conditions for efficient separation of modified oligonucleotides

The optimization of mobile phase conditions was carried out with the AAF adducted oligonucleotide (CCC CGA GCA ATC TCA AT), which has two guanine nucleobases and, as AAF is reported to form covalent bonds preferentially with guanine nucleobases, this

oligomer served as an ideal system for the development of a chromatographic system for its subsequent application to the analysis of more complex and biologically relevant mixtures. In all these cases a peak attributed to the mono AAF adducted 17-mer oligonucleotide was observed at m/z 1773.47 corresponding to -3 charge state of the isomeric compound.

3.1.1 Effect of ion pairing reagent—There is a complex relationship between retention and MS sensitivity in LC-ESI-MS/MS of oligonucleotide analysis when it comes to selections of the type and concentration of the ion pairing reagent. Lower amounts of ion pairing reagent lead to poorer retention and higher MS signal while higher amounts lead to increased retention but also signal suppression. Hence an optimal ion pairing reagent concentration in the mobile phase is required for high resolution nucleotide separation, increased oligonucleotide retention and MS sensitivity. Accordingly, various ion pairing reagents were evaluated not only for their effect on retention time and relative MS sensitivity but more importantly in terms of their ability to separate positional isomers (Figure 2).

For all these evaluations of IPRs a solution of 9.09 picomole/ μL of the AAF adducted 17 nucleotide oligonucleotide was injected multiple times onto the column and a linear gradient of 1% B per minute was used for the isomeric oligonucleotide adducts separation at a flow rate of $6\mu\text{L}/\text{min}$. The various ion pairing reagents vary many fold in their activity towards the AAF adducted oligonucleotide under similar conditions of LC-MS as illustrated in Figure 2 for the extracted ion chromatogram for the -3 charge state and are further summarized in Table 2.

In general, an increase in retention time is observed with increasing alkyl chain length or higher degree of IPR alkylation due to the increased hydrophobicity. It is also observed that the best MS sensitivity is provided by 16.5 mM *N,N*-dimethylbutylamine/ 400 mM HFIP (R_t 31.64, NL $3.93\text{E}10^7$) followed by 16.5 mM *N,N*-dimethylcyclohexylamine/ 400 mM HFIP (R_t 42.01, NL $2.15\text{E}10^7$) and 2.5 mM 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)/ 400 mM HFIP (R_t 30.89, NL $2.50\text{E}10^6$) respectively (Figure 2 (j), (i), (h)). The increased mass sensitivity in the case of ion pairing reagents which have HFIP as a buffering acid is due to the very low boiling point of HFIP (bp=58.2°C) which reduces the surface tension of the formed droplets and results in overall increase in ionization efficiency. In the case of the bicarbonate salts, MS sensitivity follows the order *N,N*-dimethylcyclohexylammonium bicarbonate (DMCyHAB) > *N,N*-dimethylbutylammonium bicarbonate (DMBAB) > triethylammonium bicarbonate (TEAB) > hexylammonium bicarbonate (HxAB). The signal suppression in the bicarbonate salts as compared to fluoroalcohols might be due to the higher conductivity of the bicarbonate anion and this increased ionization competition results in a lower MS signal. When compared to other ion pairing reagents, bicarbonate salts and in particular triethylammonium bicarbonate, form primarily -3 ion charge state for the adducted oligonucleotide upon ESI, which may be due to its higher gas-phase proton affinity. Triethylammonium acetate (TEAA) and *N,N*-dimethylcyclohexylammonium acetate (DMCyHAA) were also tested under similar conditions for AAF adducted 17 nucleotide long oligonucleotides (Figure 2 (f), (e)). Use of TEAA produced poor peak shape and lower MS signal as compared to the higher MS signal in the case of the more hydrophobic DMCyHAA.

Of highest significance in terms of the focus of this investigation was the effect of these ion pairing reagents on the separation of positional isomers of the AAF adducted oligonucleotide. It was determined that under the same chromatographic conditions the mobile phases containing the ion pairing reagents triethylammonium bicarbonate and *N,N*-dimethylbutylammonium bicarbonate were the only ones able to separate the isomeric oligonucleotides satisfactorily (Figure 2 (a), (b)). The half height method was used to

measure peak resolution (R_s) of isomeric adducts separated using TEAB as ion pairing reagent and was found to be 0.75 whereas DMBAB did not resolved peaks at half peak height.

A bicyclic amidine, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) having a very high pK_a (24.34 in acetonitrile) was also used as an ion pairing reagent along with HFIP as buffering agent. Even at a lower concentration (2.5 mM, pH 8.4, 400mM HFIP) DBU gave higher MS sensitivity but a poor separation of positional isomers (Figure 2 (h)). The effectiveness of DBU as ion pairing reagent was further probed by adding 2.5 mM of DBU to a mobile phase containing 16.5 mM TEA/400mM HFIP, which resulted in poorer separation of the positional isomers of AAF adducted oligomer in the absence of DBU (Figure S1).

Although the role of ammonium cation in these ion pairing reagents is well understood, it is the role of the counter ion, bicarbonate or acetate ion which is not clear. The counter anion definitely plays a role in the separation of positional isomers as shown in Figure 2 and most notably it is the presence of the bicarbonate ion which helps improve the chromatographic separation of isomeric oligonucleotides. The failure of IPRs with HFIP to separate positional isomers could possibly be related to the earlier reports that chromatography of unmodified oligonucleotides in case of the mobile phase containing HFIP is less dependent on base composition [39]. The detrimental effect of the HFIP on the separation of positional isomers was observed again when, upon addition of 400mM HFIP to the mobile phase containing 25mM TEAB aqueous solution, two isomers were not resolved (Figure S2).

3.1.2. Effect of ion pairing reagents concentration—Initial investigations were carried out using TEA/ HFIP which has been the gold standard in the MS analysis of oligonucleotides [40]. Varying the concentration of the triethylamine from 4 mM to 25 mM results in an increase in MS sensitivity, unstable retention times and moreover, none of these concentrations were able to resolve the positional isomers (Supplementary Figure S3). The effect of concentration of triethylammonium bicarbonate on the resolution of the positional isomers over a range of 10–25 mM was investigated next (Figure 3). Although retention time decrease was only marginal with decreasing concentration of the TEAB ion pairing reagent, a lower concentration of TEAB did result in higher MS signal. However, it was found that 25 mM TEAB concentration was optimal for the separation of the two positional isomers.

3.1.3. Effect of flow rate and linear gradients—Following optimization of the monolithic PS-DVB column and the ion pairing reagents, the chromatographic profiles of positional isomers were further evaluated as a function of gradient flow rate. At higher flow rates of 10 μ L/ minute there was no significant effect on the separation of the positional isomers. The effect of linear gradients (0.55% to 2.5% B per minute) on resolution was also evaluated at a flow rate 10 μ L/ minute and separation of the two positional isomers was found to decrease significantly (Figure S4). At 2.5% B per minute two peaks were visible but poorly resolved such that the faster chromatography was found to be detrimental for the separation of positional isomers.

3.1.4. Effect of acetate salts as mobile phase additive—It was decided further to check the effect of introduction of volatile neutral salts of acetate in the mobile phase containing 25mM triethylammonium bicarbonate on the overall chromatographic performance of the system. The selected salts, ammonium acetate, triethylammonium acetate and *N,N*-dimethyl cyclohexylammonium acetate also act as ion pairing reagents alone, thus their presence makes the mobile phase a multi ion pairing reagent (Figure 4). The overall retention and chromatographic separation were affected only slightly by the presence of ammonium acetate (5–25 mM concentration) in the presence of TEAB. Similar was the

case with triethylammonium acetate (10–25 mM concentration) but the effect was more pronounced in the case of *N,N*-dimethylcyclohexylammonium acetate where an increase in its concentration resulted in increased retention and poor peak shape. These results suggest that the ion pairing effect of TEAB is not perturbed due to the presence of lower amounts of mobile phase additives of volatile neutral salts of acetate.

3.1.5. Structural identification of positional isomers of the adducted oligonucleotide

—Structural characterization of the AAF adducted isomeric oligonucleotides was obtained by MS/MS with the spectra acquired at 30 % collision energy (Figure 5). A series of fragment ions (a – B) (derived from the 5' end of the oligonucleotides) and w (derived from the 3' end) were used to identify and confirm the backbone sequence and sites of adduct formation following the rules described by McLuckey and co-workers [41]. In the case of 25 mM TEAB it was found that the first peak corresponds to adduction at G(1) position (closest to 5' end) and the second peak corresponds to adduction at the G(2) position (Table 3). The presence of an ion at m/z 1846.60 corresponds to unadducted W_{12}^{2-} fragment ion and the peak at m/z 1805.13 corresponds to the $(a_6-B_6)^-$ fragment ion containing the adduct. Further, presence of m/z 1253.93 peak corresponds to the unadducted $(a_5-B_5)^-$ and confirms that Peak I corresponds to adduction at the G(1) position. Similarly the absence of these aforementioned ions in the spectrum of Peak II, along with the presence of the m/z 1957.07 (W_{12}^{2-}) fragment ion containing the adduct corresponds to an adduct formed at the G(2) position. The MS/MS spectrum of Peak II further shows the presence of unadducted $a_6-B_6^-$ (m/z 1584.13) and unadducted W_{11}^- (m/z 1800.00) fragment ions reconfirming that adduct is located at G(2) position in peak II.

3.2. Mapping of sites of base modification of AAF adducted oligonucleotides containing codon 135 of p53 gene

Various environmental exposures have been considered as carcinogenic in nature. The most worrisome aspect for many of the carcinomas such as bladder cancer is the fact that gene mutations corresponding to bladder tumor are detected at advanced stages only. Recently a TGC to TTC mutation of exon 5, codon 135 of p53 gene has been reported for grade G2b bladder cancer [42], pointing to the need of monitoring tools for continuous assessment of carcinogenic activity around 'hot-spots' such as codon 135 of p53 gene for early detection of cancer.

A proof of concept study was carried out using the analytical platform based on monolithic PS-DVB column and IP-RP-LC-ESI-MS/MS for the mapping of a 15 base pair oligonucleotide containing the codon 135 of p53 gene for the presence of carcinogen AAF adduct. A mixture of AAF adducted oligonucleotides (TG TTT TGC¹³⁵ CAA CTG G) was chromatographically separated using optimized mobile phase conditions over a PS-DVB monolithic capillary column and using TEAB as an ion pairing reagent (Figure 6). The panels show the extracted ion chromatograms $[M-3H]^{3-}$ for the singly, bis-, tris-, and tetra AAF adducted oligonucleotides at m/z 1598.038, 1671.704, 1745.401, and 1819.098, respectively eluting between 24.11 and 47.79 minutes. It was interesting to note that for every additional AAF adduct onto the backbone, the retention time increased due to the additional π - π stacking interactions between the aromatic rings of the PS-DVB support material and the aromatic ring of the adducts such that it took 8 additional minutes or 8% more methanol for elution. It is also interesting to observe that for the tetra AAF adducted oligonucleotide (TG TTT TGC¹³⁵ CAA CTG G) eluting at 47.79 minutes only one chromatographic peak was produced suggesting that all G nucleobases positions are occupied. Under the present experimental conditions, no T, C or A adducts were observed.

In the case of the mono- AAF adducted oligonucleotides, four chromatographic peaks corresponding to m/z 1598.038 $[M-3H]^{3-}$ eluted between 24.11 and 27.42 minutes, two of which were partially resolved (24.91 minutes) and the other two are highly resolved (24.11 and 27.42 minutes). The MS/MS fragmentation of the parent ions of these isomers (m/z 1598.038 $[M-3H]^{3-}$) was obtained to determine their structural identity (Figure S5).

The presence of the ion of m/z 1948.26 in the MS/MS spectrum of Peak IV at retention time 27.42 minutes corresponds to the unmodified $(a_7-B_7)^-$ ion while the fragment m/z 979.81 indicates that there is no modification in the W_3^- ion. The peak at m/z 1092.71 corresponds to unadducted W_7^- ion, that of m/z 1895.76 to unadducted W_6^- ion while the presence of 1249.3 corresponds to a modified $(a_8-B_8)^-$ fragment. Finally, a prominent peak at m/z 1393.96 corresponds to an adducted $(a_9-B_9)^-$ ion thus confirming that Peak IV has an AAF adduct at G (2) position (TG TTT TGC¹³⁵ CAA CTG G). It should be pointed out that the six positional isomers of the bis- adducted oligonucleotide were only partially separated as shown in Figure 6. The low resolution or inability to separate the positional isomers for adducted oligonucleotide could be due to the closely situated guanine nucleobases such as G(3) and G(4) situated next to each other in the present case [43].

For each of the Peaks of the positional isomers of AAF modified oligonucleotide the diagnostic pairs of fragment ions $[w_n]^-$ and $[a_n - B_n]^-$ obtained from MS/MS spectrum were used to confirm the site of adduction.

3.3. Profiling of a complex mixture containing p53 gene fragments

To further test the general applicability and robustness of our developed platform, profiling of a complex oligonucleotide mixture was performed. The objective was to test the PS DVB monolithic column based ESI-LC-MS/MS platform for the separation of diverse oligonucleotide mixture.

This complex mixture consists of synthetic AAF modified and unmodified oligonucleotides of different length and sequence. It contains seven unmodified 15 mer long oligonucleotides, two AAF modified 15 mer long oligonucleotides, In addition to these it also consists of a AAF modified 12 mer, an unmodified 17 mer and a AAF modified 18 mer long oligonucleotide (Table S1). Among the AAF modified oligonucleotides the more important sequences are the ones which are from p53 gene containing codon 135 (TG TTT TGC¹³⁵ CAA CTG G), codon 157 (C CGC GTC¹⁵⁷ CGC GC) and codon 248 (ATG AAC CGG²⁴⁸ AGG CCC). The listed codons, especially codon 157 & codon 248 are the 'hot-spot' which could be adducted by multiple genotoxin chemicals. The mutations in and around these 'hot-spots' are known to be carcinogenic in nature, thus calling for the need for technologies which can assist in early screening of the genotoxin adduct at mutational 'hot-spots'.

The mixture components were separated and sequenced over a 90 minute linear gradient run (0.5%B per minute) using the developed LC-ESI-MS/MS method (Figure S6, Figure 7).

As evident from Figure 7, the LC-MS chromatogram of this complex mixture could be divided into 3 segments. In the first 25 minutes of the chromatographic run unmodified oligonucleotides elute according to their composition and hydrophobicity. In the second segment (25.00–50 minute) mono or bis- AAF adducted oligonucleotide elutes and finally, in last segment (50.00 – 100.00 minute) is populated by tris-, and tetra AAF adducted oligonucleotide adducts.

While the primary components of the mixture were well-separated, some of the positional isomers were not sufficiently resolved, primarily those containing closely situated adducts. The described profiling of complex mixture could be made more useful especially for

biological mixtures comprised of unknown oligonucleotides by developing software for nucleic acids characterization and de novo sequencing by tandem mass spectrometry.

Conclusions

Efficient high resolution chromatographic separation and online sequencing of modified oligonucleotides is highly desired in order to study directly the relationship between activated forms of carcinogen and gene 'hotspots'. In this regard, a reversed-phase ion-pair LC method using a monolithic column coupled to an ion trap mass spectrometer has been developed for the separation and sequencing of isomeric adducted oligonucleotides individually or in a mixture.

Such a model study using synthetic oligonucleotides on the IP-RP-LC-ESI-MS/MS platform helps to identify the best chromatographic conditions which could in future be extrapolated to the analysis of genomic DNA digests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Optimization of mobile phase conditions for efficient separation and sequencing of isomeric oligonucleotide adducts.

Mapping of AAF adducted 15 mer long oligonucleotide containing codon 135 of p53 gene.

Profiling of complex mixture comprising of unadducted, singly or multiple AAF adducted oligonucleotides of p53 gene fragments.

Figure 1.
Various amines used as ion pairing agents

Figure 2.

Effect of ion pairing reagent on IP-RP-LC-ESI-MS separation of AAF adducted oligonucleotide [CCC CGA GCA ATC TCA AT].

The figure shows extracted ion chromatograms [EIC] for the -3 charge state of 17 mer AAF adducted oligonucleotide, linear gradient 1%B per minute.

(a) 25 mM triethylammonium bicarbonate (b) 25 mM N,N dimethylbutylammonium bicarbonate (c) 25 mM N,N hexylammonium bicarbonate (d) 25 mM N,N dimethylcyclohexylammonium bicarbonate (e) 25 mM N,N dimethylcyclohexylammonium acetate (f) 25 mM triethylammonium acetate (g) 25 mM trans-N,N'-dimethylcyclohexane-1,2-diamine/ 100 mM HFIP (h) 2.5 mM 1,8-

Diazabicyclo[5.4.0]undec-7-ene (DBU)/ 400 mM HFIP (i) 16.5 mM N,N-dimethylcyclohexylamine/ 400 mM HFIP (j) 16.5 mM N,N-dimethylbutylamine/400 mM HFIP (k) 16.5 mM hexylamine/ 400 mM HFIP (l) 16.5 mM tripropylamine/ 400 mM HFIP (m) 16.5 mM triethylamine/ 400 mM HFIP (n) 25 mM ammonium acetate

Figure 3. Effect of concentration of TEAB ion pairing reagent on the separation of positional isomers of AAF adducted oligonucleotide [CCC CGA GCA ATC TCA AT]. The figure shows extracted ion chromatogram [EIC] for the -3 charge state of 17 mer AAF adducted oligonucleotide.

Figure 4.

Effect of mobile phase additives on TEAB separation of positional isomers of AAF adducted 17 mer oligonucleotide [*CCC CGA GCA ATC TCA AT*]. The figure shows extracted ion chromatogram [EIC] for the -3 charge state of 17 mer AAF adducted oligonucleotide.

(a) 25 mM TEAB + 25 mM N,N dimethyl cyclohexylammonium acetate (b) 25 mM TEAB + 10 mM N,N dimethyl cyclohexylammonium acetate (c) 25 mM TEAB + 5 mM N,N dimethyl cyclohexylammonium acetate (d) 25 mM TEAB + 25 mM TEAA (e) 25 mM TEAB + 10 mM TEAA (f) 25 mM TEAB + 25 mM Ammonium acetate (g) 25 mM TEAB + 10 mM Ammonium acetate (h) 25 mM Triethylammonium bicarbonate

Figure 5.

Structural identification of isomeric oligonucleotide peaks

(a) The figure shows extracted ion chromatogram [EIC] for the -3 charge state of 17 mer AAF adducted oligonucleotide, 25 mM triethylammonium bicarbonate as ion pairing reagent, linear gradient 1% B per minute.

(b) (c) Structural identification based on fragment ion spectra of the m/z 1773.47 (singly adducted AAF 17 mer oligonucleotide obtained from LC-MS/MS by monolithic PS-DVB column).

Figure 6.

IP-RP-LC-ESI-MS/MS of sites of base modification of AAF adducted oligonucleotide containing codon 135 of p53 gene (TG TTT TGC¹³⁵ CAA CTG G).

Mobile phase A: 25 mM triethylammonium bicarbonate as ion pairing reagent, linear gradient 1% B per minute.

The figure shows extracted ion chromatogram [EIC] for the -3 charge state of 15 mer AAF adducted oligonucleotide. (Figure S5, Table S1)

Figure 7.

IP-RP-LC-ESI-MS/MS separation of oligonucleotide mixture. The chromatographic separation is divided into 3 segments and the figure shows extracted ion chromatogram [EIC] (a) Segment1(5–25 minutes): separation and sequencing of unmodified oligonucleotides and AAF adducted 12 mer oligonucleotide (b) Segment1(25–50 minutes): separation and sequencing of singly AAF adducted oligonucleotides (c) Segment1(50–100 minutes): separation and sequencing of bis-, tris-, tetra adducted AAF adducted oligonucleotides. Underlined Red color guanine nucleobase indicates the presence of AAF adduct. (Figure S6, Table S2)

Table 1

F_M is the mobile phase flow rate, η , viscosity of the mobile phase (50/50 methanol/water), Δp is the pressure drop across the PS-DVB monolithic capillary column, Total porosity $e_T = V_T/V_g$, where V_T is the retention volume of an unretained small molecule, 2'-deoxyadenosine ($F.W = 251.2$ g/mol) in 50/50 methanol/water as the mobile phase in 0.25X95 mm PS-DVB monolithic capillary column and V_g is the is the geometrical volume of the 0.25X95 mm empty capillary column. e_o is interstitial (flow-through pore) porosity is defined as V_e/V_g where V_e is the retention volume of the excluded molecular mass ($MM > 25000$) in 50/50 methanol/water as the mobile phase in 0.25X95 mm empty capillary column. The inner (mesopore) porosity is defined as $e_i = (V_T - V_e)/V_g$. K_F column permeability, d_p mean particle diameter. $K_F = F_m \cdot \eta \cdot L / \Delta p \cdot \pi \cdot r^2$, $d_p = (1 - e_o) \cdot 180 K_F / e^3_o$

Monolithic column <i>mm</i>	Mobile phase, F_M	Viscosity [mobile phase] η , (Pa.s)	Flow rate $\mu L/min$	Back pressure Δp , (Pa)	V_c Empty column μL	e_o interstitial (flow-through pore) porosity	e_i inner (mesopore) porosity	K_F column permeability <i>m</i>	d_p pore size [mesopore] μm
PS-DVB 0.25X95	50/50 methanol / water	1.5×10^{-3}	6.0	98×10^5	4.66	0.82	0.08	2.96×10^{-14}	0.709

Table 2

Ion pairing reagent/ concentration	Pka (Amine)	Boiling Point (°C) (Amine)	Retention time	Positional isomers?
25 mM Triethylammonium bicarbonate	10.72	89	25.10 26.07	Yes
25 mM N,N dimethyl butylammonium bicarbonate	10.06	93.6	31.13 32.13	Yes
25 mM Hexylammonium bicarbonate	10.56	131–132	54.26	No
25 mM N,N dimethyl cyclohexylammonium bicarbonate	10.38	158–159	41.53	No
25 mM Triethylammonium acetate	10.72	89	25.46	No
25 mM N,N dimethyl cyclohexylammonium acetate			40.78	No
25 mM <i>trans</i> -N,N'-Dimethylcyclohexane-1,2-diamine / 100 mM HFIP		78–80	38.24	No
25 mM Triethylamine/ 2.5 mM 1,8-Diazabicyclo[5.4.0]undec-7-ene, (DBU)/ 100 mM HFIP			26.66	No
2.5 mM 1,8-Diazabicyclo[5.4.0]undec-7-ene, (DBU)/ 100 mM HFIP	12.34	80–83	30.89	Partially
16.5 mM N,N dimethyl butylamine/ 100 mM HFIP	10.06	93.6	33.04	No
16.5 mM N,N dimethyl cyclohexylamine/ 100 mM HFIP			42.01	No
16.5 mM Hexylamine/ 100 mM HFIP	10.56	131–132	47.78	
16.5 mM Tripropylamine/ 100 mM HFIP	10.65	155–158	43.26	No
16.5 mM Triethylamine/ 400 mM HFIP			30.66	
16.5mM Triethylamine/ 100 mM HFIP			22.05	No
25 mM ammonium bicarbonate				

Table 3

Assignment of fragment ions of the singly adducted AAF oligonucleotide (CCC CGA GCA ATC TCA AT), m/z 1773.47 obtained from online LC-MS/MS by monolithic PS-DVB column.

Ion assignment	Peak I m/z	Peak II m/z
W_2^-	634.40	634.53
W_3^-	947.67	947.67
W_4^-	1236.809	1236.809
W_5^-	1540.93	1541.07
W_6^-	1830.191	1830.191
W_7^{2-}	1067.194	1067.194
W_8^{2-}	1223.47	1223.80
W_9^{2-}	1379.93	1380.47
W_{10}^{2-}	1524.93	1525.07
W_{11}^{2-}	1689.6	1800.00
W_{12}^{2-}	1846.60	1957.07
$(a_{13}-B_{13})^{2-}$	1978.781	1978.781
$(a_{12}-B_{12})^{2-}$	1834.235	1833.47
$(a_{11}-B_{11})^{2-}$	1682.089	1682.089
$(a_{10}-B_{10})^{2-}$	1525.00	1525.00
$(a_9-B_9)^{2-}$	1367.53	1368.876
$(a_8-B_8)^{2-}$	1223.73	1223.13
$(a_7-B_7)^-$	1059.24	1897.00
$(a_6-B_6)^-$	1805.13	1584.13
$(a_5-B_5)^-$	1253.93	1254.00
$(a_4-B_4)^-$	964.67	964.67
$(a_3-B_3)^-$	675.53	675.40