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Development of a headspace gas chromatography method to determine residual aliphatic amines in oligonucleotides

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Abstract

A headspace gas chromatographic method has been developed for analysis of aliphatic amines in oligonucleotides. The linearity was improved by including ammonia in the sample vials. Also, carryover of longer chain primary amines, hexylamine and octylamine, was substantially reduced by cleaning the injection syringe with ammonia and water vapor between sample injections.

Keywords: Aliphatic amines; Headspace gas chromatography

1. Introduction

Residual organic compounds need to be determined in many finished products. In particular, all solvents and reagents used in the manufacture of pharmaceuticals must be determined. Considerable literature exists describing methods and measurement requirements [1–6]. The primary method for determination of residual organics is headspace gas chromatography (HS-GC). HS-GC requires a minimum of operator effort and is quantitative.

A method needed to be developed for analysis of aliphatic amines in synthetic oligonucleotides. While determination of most solvents by HS-GC has become routine, aliphatic amines pose a challenge because of the high activity of aliphatic amines. A limited number of publications describe HS-GC determination of aliphatic amines [7,8]. The purpose of this article is to describe a HS-GC method for the determination of residual aliphatic amines in oligonucleotide active pharmaceutical ingredients (API). The method of Maris et al. [8] was adapted for quantitation of residual volatile compounds in oligonucleotides. The linearity was improved and carryover reduced.

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2. Experimental

2.1. Chemicals and samples

All chemicals were purchased from Aldrich or VWR Scientific and used as received. Recovery was measured using two phosphorothioate oligonucleotides synthesized by the DowPharma research group.

2.2. Instrument conditions

Table 1 lists the instrumental conditions.

2.3. Preparation of samples, standards, and blanks

The pH and ionic strength were controlled by adding an aqueous solution of 29.4 wt.% K_3PO_4 and 7.11 wt.% NaCl to each vial. Also, as described below, an ammonia solution containing (0.1 M $NH_4H_2PO_4$, 0.63 M NH_4Cl) was added to each vial.

Sample vials were prepared by weighing 25 ± 5 mg of oligonucleotide into a vial. 0.334 mL of ammonia solution was added followed by 1.05 mL (1.35 g) of K_3PO_4/NaCl solution. The vial was immediately crimped closed.

Table 1
Instrument conditions

Sampler	CTC/LEAP combiPAL
Syringe	2.5 mL gastight, 23 gauge needle, Microliter Analytical L120.0004
Vial	22 mm × 38 mm, MicroLiter Analytical 20-5000
Septum and seal	Teflon/molded butyl rubber with steel seal, MicroLiter Analytical 20-0040 M
Incubation time	15 min
Syringe fill speed	50 μL/s
Incubation temperature	80 °C
Pull up delay	5 s
Syringe temperature	85 °C
Injection speed	300 μL/s
Agitation speed	350 rpm
Injection volume	2 mL
Chromatograph	Agilent 6890
Capillary column	Varian/Chrompack CP7447 CP-Volamine 30 m × 0.32 mm (film thickness not specified)
Inlet liner	Restek 20782-211.5 with wool removed
Oven program	80 °C, 1 min, 20 °C/min to 260 °C
Injector	Split, 15.4:1, 200 °C
Column flow	1.9 mL/min He, constant flow mode
Detection	Flame ionization detection (FID)
Detector temperature	250 °C
Detector gas flows	40 mL/min H_2, 400 mL/min air, constant make-up of 20 mL/min nitrogen

Standard solutions were prepared in the ammonia solution in order to keep the aliphatic amines at a pH below the pK_a of the aliphatic amines (∼10.4). Standard vials were prepared by adding 0.334 mL of the standard solution followed by 1.05 mL of K_3PO_4/NaCl solution.

Blank vials were prepared by adding 0.334 mL of ammonia solution followed by 1.05 mL of K_3PO_4/NaCl solution.

3. Results and discussion

3.1. Initial results

When the method of Maris et al. [8] was initially applied to quantitation of aliphatic amines in oligonucleotides, the procedure described in the publication was followed; the ionic strength was maximized using a combination of 0.32 g of NaCl and 0.1 g of K_2SO_4; 1 mL of a buffer of 0.1 M HCl was added where the HCl buffer was used to preserve the aliphatic amine standards; the pH was increased by adding 0.1 mL of 50 wt.% NaOH to the vials. Measurements of linearity for diethylamine (DEA) and pyridine using these conditions are summarized in Fig. 1. The results for pyridine exemplify the desired result; the response/concentration is constant with concentration. For DEA, a significant decrease in response/concentration with a decrease in concentration was observed.

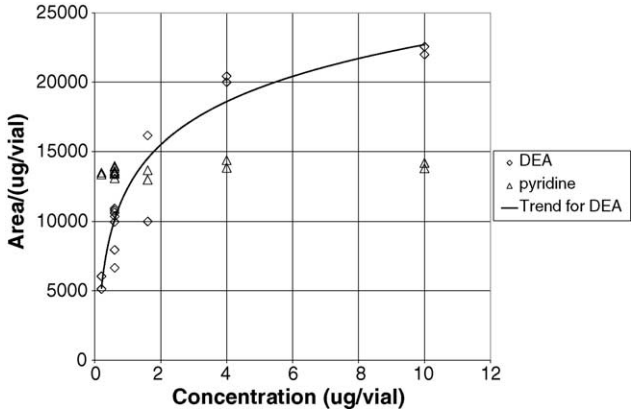

Fig. 1. Response/concentration vs. concentration without ammonia added to the sample vial. (The line was drawn to guide the eye through the results for DEA and does not represent a regression fit of the data.)

Based on the suggestion of Maris et al. [8] that peak shape was improved by adding ammonia to the sample, as well as literature reports where the recovery of aliphatic amines from GC columns was increased by adding ammonia to the carrier gas [9,10], ammonia was added to the vials via the procedure given in the experimental section. This results in 0.24 mmol of ammonia per vial.

Measurements of response per concentration for DEA as a function of DEA concentration with ammonia present in the sample vial are given in Fig. 2. Significant improvement in linearity was observed when ammonia was added. Additional experiments found that increasing the amount of ammonia to more than 0.24 mmol/vial did not improve performance and that decreasing the amount to 0.08 mmol/vial resulted in a decrease in response/concentration for DEA at lower DEA concentrations. It is hypothesized that the presence of the ammonia in the headspace masks active sites in the headspace syringe although it may also improve recovery from the inlet of the GC. The addition of ammonia to the sample did not influence the shape of the aliphatic amine peaks.

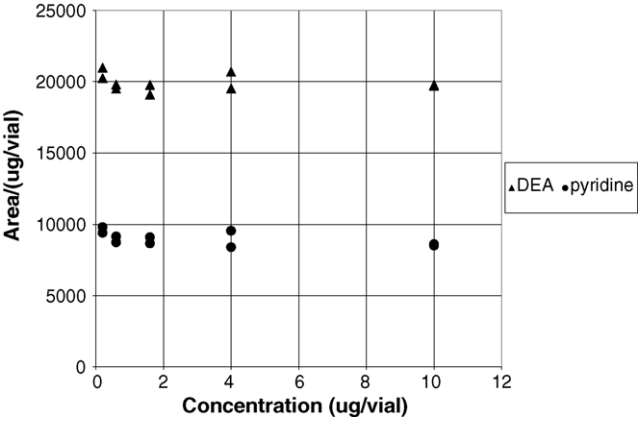

Fig. 2. Response/concentration vs. concentration with ammonia added to the sample vial.

Table 2
The effect of flushing the syringe with vapors from a blank vial

	Ethylamine	DEA	Butylamine	TEA	Hexylamine	Octylamine
Carryover with flush step						
1st vial	ND	0.7%	1.0%	1.5%	0.6%	3.6%
2nd vial	ND	0.8%	1.1%	1.5%	0.8%	1.0%
Carryover without flush step						
1st vial	ND	1.5%	1.0%	1.3%	7.0%	18.0%
2nd vial	ND	ND	0.8%	0.7%	1.0%	8.0%

ND = not detected. (Table gives the size of the peak detected in a blank following a standard injection as a percentage of the peak area in the standard.)

Table 3
Summary of repeatability and LOQ measurements

	Ethylamine	DEA	Butylamine	TEA	Hexylamine	Octylamine
RSD	2.3%	3.2%	4.2%	2.0%	1.6%	2.1%
LOQ (μg/g)	5	1.2	Not measured	0.7	0.8	0.9

Repeatability was measured from eight injections of a standard that corresponded to 25 μg of analyte/g of oligonucleotide.

3.2. Reduction of carryover

For the primary amines, carryover was observed and limited the accuracy of headspace measurements. The software provided by LEAP flushes the syringe with nitrogen after making the injection. This software was modified to perform the flush while the next sample is incubating. Also, the syringe was flushed with the vapor from a blank vial containing the ammonia and salt solutions. A blank vial was stored in the incubator during the entirety of the measurement sequences. The syringe sampled the headspace of this blank vial and was then flushed with nitrogen three times during the 15 min period that the next vial was incubating. The efficacy of this extra flush step in reducing carryover is indicated by the data summarized in Table 2. The extra flush step reduced the amount of carryover for the longer aliphatic amines, hexylamine and octylamine, by more than a factor of four. The amount of carryover was observed to vary with the particular syringe used for sampling. Carryover generally increased with syringe usage. When this purge step was used, some syringes gave no carryover at all. Carryover was not observed regardless of how the syringe was purged for analytes that were not amines.

3.3. Summary of method performance

The performance of the method is summarized in Tables 3–5 and Fig. 3. Fig. 3 displays an example chromatogram of a standard that contains each aliphatic amine at a concentration that corresponds to 25 μg of each amine per gram of oligonucleotide. Table 3 summarizes measurements of system precision and limit of quantitation (LOQ). LOQ was estimated as the analyte concentration needed to obtain a peak height 10 times the baseline noise. Table 4 summarizes linearity measurements for a series of aliphatic amines. Similar linearity data (not presented here) were also obtained for the other analytes listed in Table 5 that are not aliphatic amines. Table 5 summarizes the recovery measurements. Quantitation was by external standardization.

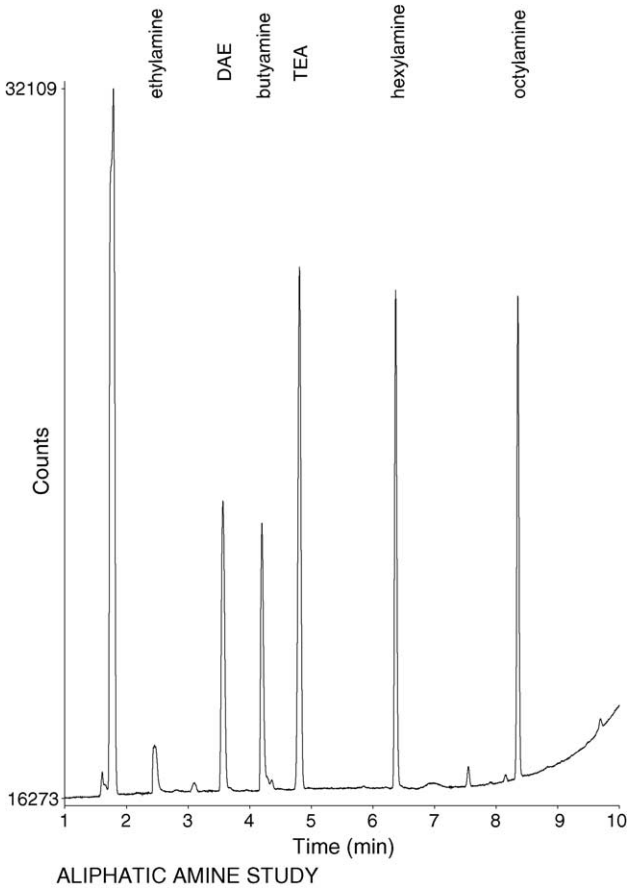

Fig. 3. Example chromatogram of a standard with a concentration of each analyte that corresponds to 25 μg/g in oligonucleotide.

Table 4
Summary of linearity determination from measurements at five different concentrations from 0.9 to 50 μg/g

Compound	R^2	Slope	Standard error	Intercept	Standard error	Area of lowest concentration standard	Standard error of the peak estimate
Ethylamine	0.997	3637	54	−500	104	2465	140
DEA	0.997	14267	231	−934	483	11495	645
Butylamine	0.995	10846	223	−433	496	9569	663
TEA	0.999	21825	211	−530	466	19166	623
Hexylamine	0.999	16033	136	−1254	301	13045	402
Octylamine	0.996	13894	246	−977	552	11172	737

Duplicate measurements were made at each concentration except 25 μg/g for which there were eight measurements.

Table 5
Summary of recovery measurements

Compound	Concentration (μg/g)	Oligonucleotide 1 (%)	Oligonucleotide 2 (%)
Ethanol	49	91	99
Ethanol	98	96	101
Ethanol	195	94	97
Pyridine	49	84	91
Pyridine	97	87	92
Pyridine	193	86	90
DEA	12	96	97
DEA	23	96	98
DEA	46	99	99
Hexylamine	13	92	97
Hexylamine	25	92	96
Hexylamine	50	96	98
Acetonitrile	48	95	98
Acetonitrile	97	97	98
Acetonitrile	192	97	96
Toluene	51	98	100
Toluene	103	99	98
Toluene	210	101	98
TEA	12	99	101
TEA	24	100	101
TEA	48	101	100

4. Conclusion

Previous reports provided a sound basis for developing a method to quantitate aliphatic amines in oligonucleotides, or other APIs [8]. Significant improvement in linearity was realized by adding ammonia to the sample vial. Carry-over for hexylamine and octylamine was reduced by a factor of at least four by flushing the syringe with ammonia and water vapor instead of just purging the syringe with nitrogen.

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