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Subject: Evaluation of e-cigarettes

Background: The Center for Drug Evaluation and Research through the Office of Compliance (OC) has requested that the Division of Pharmaceutical Analysis (DPA) evaluate two brands of electronic cigarettes (e-cigarettes) for nicotine content and other impurities. An e-cigarette is advertised as an alternative to smoked tobacco products. It is a battery-powered device that provides inhaled doses of nicotine by delivering a vaporized propylene glycol/nicotine mixture as shown in Figure 1. The Center is concerned that in addition to nicotine delivery, the vapor may also provide other potentially harmful volatile components. DPA was asked to quantitate the amount of nicotine present in each brand and to evaluate each brand for the presence of tobacco specific nitrosamines (TSNA), certain tobacco specific impurities, ethylene glycol (EG) and diethylene glycol (DEG).

Test Products: Njoy e-cigarette (Reference 1) with various cartridges

Smoking Everywhere Electronic Cigarette (Reference 2) with various cartridges

Nicotrol Inhaler, 10mg cartridge was used as a control for some test methods

Conclusions: Nicotine is present in both products. The Smoking Everywhere Electronic Cigarette cartridges listed as containing no nicotine in some cases had very low amounts of nicotine present. Tobacco specific nitrosamines and tobacco specific impurities were detected in both products at very low levels. DEG was identified in one cartridge, Smoking Everywhere 555 High. See Table 1 for results of analyses of entire cartridges after extraction.

A sparging apparatus (see figure in Attachment A) and headspace GC (HS-GC) analysis were utilized to simulate actual use of these products. With the sparging apparatus, nicotine was detected in cartridges claiming to contain nicotine and quantitated by LC-UV; cotinine was also found in some products by this procedure. Repeat testing of 3 different cartridges with the same label (menthol high) gave varying results from 26.8 to 43.2 mcg nicotine/100 mL puff. HS-GC detected nicotine in both products and β-Nicotyrine was detected in all Njoy cartridges (see Table 2).

Experimental: See Attachment A for further experimental details.

- Nicotine content was analyzed by HPLC-UV and GC-MS. Quantification was done by HPLC-UV using two different extractions: a methanol extraction, and a 10% acetonitrile/ 1% phosphoric acid in water extraction.
- Tobacco specific impurities and diethylene glycol were analyzed by GC-MS.
- Diethylene glycol presence was confirmed with proton NMR.
- Detection of nicotine and tobacco specific impurities during use of these products was estimated by simulating use temperatures and analyzing volatiles using head space GC-MS (HSGC-MS) and utilizing a sparging apparatus (see figure in attachment).

Results and Discussion:

Whole Cartridge: Nicotine content by HPLC-UV

Results, similar for both methanol extraction and 10% acetonitrile/1% phosphoric acid in water extractions, are shown in Table 1.

Whole Cartridge: Tobacco Specific Nitrosoamines by LC-MS/MS

The four major TSNAs include: N-nitrosonicotine (NNN), N-nitrosoanabasine (NAB), N-nitrosoanatabine (NAT) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were analyzed for using LC-MS/MS and the results are shown in Table 1.

Whole Cartridge: Tobacco Specific Impurities by GC-MS and GC-MS/MS

Nicotine was detected in both products in all cartridges including samples identified as containing no nicotine. Samples were screened for possible tobacco specific impurities: cotinine, nicotine-N-oxide, nornicotine, anatabine, anabasine, pseudooxynicotine, myosmine, β-nicotyrine, and 1-methyl-3-nicotinoylpyrrolidine (MNP). Nicotine-N-oxide, nornicotine, anatabine, pseudooxynicotine and MNP were not observed in any of the samples. Results from cotinine, anabasine, myosmine, and β-nicotyrine are shown in Table 1.

Whole Cartridge: Diethylene Glycol by GC-MS

Diethylene Glycol was detected in one sample (Smoking Everywhere 555 High cartridge) at approximately 1%.

Simulated Use: Nicotine and tobacco Specific Impurities by Head Space GC-MS (HSGC-MS) and sparging apparatus

HSGC is likely to be less sensitive than the GC-MS technique that takes advantage of injecting all of the soluble components and then volatilizing them at 280 °C; however, the head space analyzer can be set to a specific temperature to mimic what may be volatilized during use of the products.

The temperature of the heating element in each e-cigarette was determined by inserting a thermocouple and then activating the e-cigarette by drawing air through it. These temperatures ranged from 40 to 65°C. HSGC-MS analysis was conducted at 60°C to simulate the temperature that would be encountered during activation of an e-cigarette. Nicotine was detected in both products for all cartridges containing low, medium and high levels of nicotine but was not

observed in cartridges identified as containing no nicotine. Screening for the possible tobacco specific impurities cotinine, nicotine-N-oxide, nornicotine, anabasine and myosmine was negative. β-Nicotyrine was detected in all Njoy cartridges but was not detected in the Smoking Everywhere cartridges. The sparging apparatus was used to quantify the amount of nicotine released during use of these electronic cigarettes (Table 2). Levels found were consistent with the labeling (low, medium and high); however, the cartridge labeled "no" still delivered some nicotine. The cartridges labeled "high" delivered more nicotine than the approved Nicotrol product. Repeat testing of 3 different cartridges with the same label (menthol high) gave varying results from 26.8 to 43.2 mcg nicotine/100 mL puff.

References:

(1) http://www.njoythefreedom.com/

(2) http://www.smokingeverywhere.com/

(3) C.N. Man, L.H. Gam, S. Ismail, R. Lajis, R. Awang, J. Chromatogr. B 844 (2006) 322–327.

(4) S. S. Yang et al., J. Chromatogr. A, 942 (2002) 33-39.

(5) USP 31/NF 26, Official 12/1/08-4/30/09 NF Monographs: Diethylene Glycol Monoethyl Ether: Assay; pg 1126

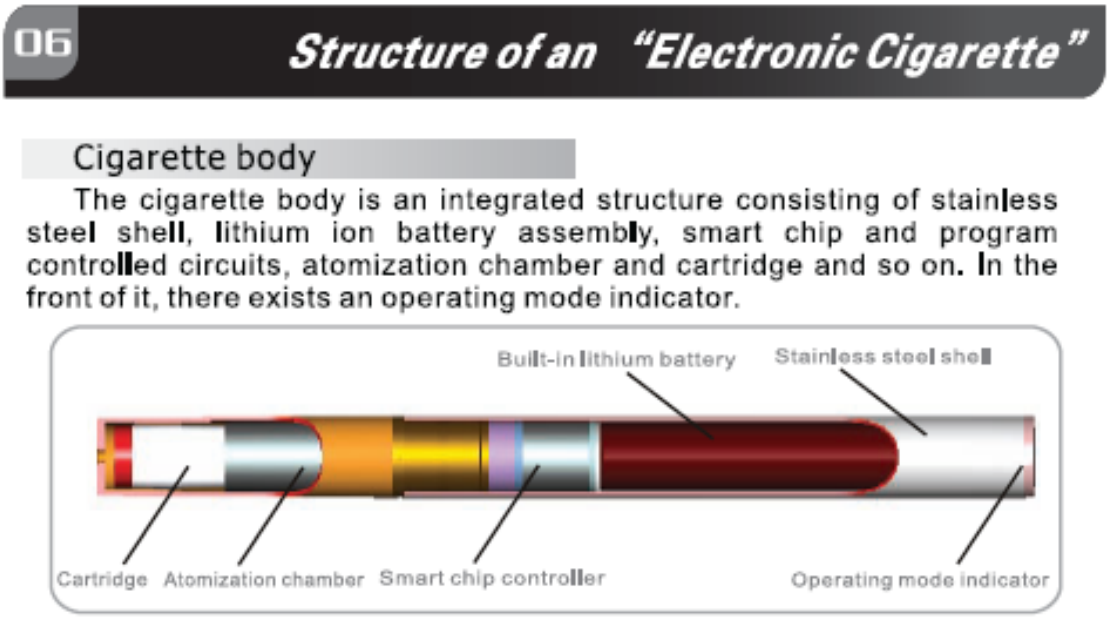

Figure 1: E-cigarette component diagram. Nicotine and/or other flavorants are housed in the white cartridge shown at the left.

Table 1. Results on analyses of whole cartridges

Sample	Tobacco specific nitrosamines (TSNA)[A]				mg nicotine/cartridge by HPLC-UV			Identification of possible tobacco specific impurities and DEG by GC-MS/MS[B]				
					10% ACN and 1% PA in water extraction		methanol extraction					
Smoking Everywhere e-cigarettes	NAB	NAT	NNK	NNN	fiber absorbent	plug & wrapper	fiber absorbent	DEG	Cotinine	Anabasine	Myosmine	β-nicotyrine
555 High					4.90	1.80	5.98	D	D	D	D	D
Cherry HIGH					4.88	0.86	5.50	ND	D	ND	D	D
Tobacco Original MED	ND	ND	D	D	4.44	1.03	5.15	ND	D	D	D	D
Menthol HIGH					4.02	0.71	4.23	ND	D	D	D	D
Tobacco Original LOW	ND	ND	D	D	2.93	0.51	2.65	ND	D	D	D	D
Menthol MED					2.26	0.31	2.71	ND	D	D	D	D
Apple LOW	ND	ND	ND	ND	1.63	0.61	1.78	ND	D	D	D	D
Menthol LOW					1.42	0.30	1.57	ND	D	ND	D	D
Vanilla LOW	ND	ND	ND	ND	1.18	0.34	0.96	ND	D	D	D	D
Tobacco Original NO	ND	ND	ND	ND	0.04	0.00	0.03	ND	ND	ND	ND	ND
Vanilla NO					0.04	0.01	0.05	ND	ND	ND	ND	ND
Cherry NO					0.01	0.00	0.07	ND	ND	ND	ND	ND
Chocolate NO	ND	ND	ND	ND	0.01	0.00	0.03	ND	ND	ND	ND	ND
Apple NO	ND	ND	ND	ND	0.00	0.00	0.00	ND	ND	ND	ND	ND
Njoy e-cigarettes												
Menthol high	D	D	D	D	6.66	1.97	6.76	ND	D	D	D	D
Regular medium	D	D	D	D	4.09	1.58	4.31	ND	D	D	D	D
Menthol medium	D	D	D	D	3.98	1.46	4.77	ND	D	D	D	D
Regular low					3.35	1.56	5.16	ND	D	D	D	D
Control Sample – Nicotrol 10 mg cartridge Mfgr. specification									(NMT 0.5%)		(NMT 0.5%)	(NMT 0.5%)

D= detected, , ND= not detected NAB = N-nitrosoanabasine (LOQ = 21 ppb); NAT = N-nitrosoanatabine (LOQ = 21 ppb); NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (LOQ = 75 ppb); NNN = N-nitrosonornicotine (LOQ = 24 ppb)

[A] analyte was detected but at a level less than the limit-of-quantitation. Open boxes indicate the sample was not available for testing.

DEG = diethylene glycol

[B] Limit of detection Cotinine 20 ppb. Anabasine 10 ppb; myosmine 69 ppb; β-nicotyrine 170 ppb – present but at less than the level of the Nicotrol specification

Table 2. Results on simulated use of electronic cigarettes

	Sparging Apparatus followed by LC-UV analysis		**Head Space GC-MS @ 60 deg C***	
Sample	mcg nicotine/100mL puff	mcg cotinine/100 ml puff (LOD ~0.03)	β-nicotyrine	nicotine
Smoking Everywhere e-cigarettes				
555 High	31.5	0.4		
Cherry HIGH			ND	D
Tobacco Original MED	15.7	trace	ND	D
Menthol HIGH				
Tobacco Original LOW				
Menthol MED			ND	D
Apple LOW			ND	D
Menthol LOW	9.9	ND	ND	D
Vanilla LOW			ND	D
Tobacco Original NO			ND	ND
Vanilla NO			ND	ND
Cherry NO			ND	ND
Chocolate NO			ND	ND
Apple NO	0.35	ND	ND	ND
Njoy e-cigarettes				
Menthol high	**43.2, 34.9, 26.8**	**trace**	**D**	**D**
Regular medium			D	D
Menthol medium	10.6	ND	D	D
Regular low				
Control (specs)				
Nicotrol 10mg cartridge	15.2			

DEG = diethylene glycol

D= detected, ND= not detected

* Presence of tobacco specific impurities cotinine, nicotine-N-oxide, nornicotine, anabasine and myosmine was negative at 60 deg C.

Open boxes indicate the sample was not tested.

Experimental Details:

Whole Cartridges: Detection and Quantitation of Nicotine by HPLC-UV
Smokeless tobacco cartridges were extracted and analyzed using two different procedures:

- Methanol extraction and USP analytical procedure: cartridge components were weighed, extracted with methanol and reweighed. An aliquot of the methanol extract was diluted with mobile phase and analyzed by HPLC-UV following USP 31 p 2801 Nicotine Transdermal System Assay procedure.

- Extraction with (b) (4) as described in NDA 20-714, Phamacia & Upjohn method "Nicotine, Content Uniformity, Identification and Determination of Nicotine Related Substances in Nicotine Inhaler 10 mg" method NM-046-6 dated 1999-07-16

Whole Cartridges: Analysis of Tobacco Specific Nitrosamines by HPLC-MS/MS

Analysis was done using a variation of the method published by Wu, et al. using HPLC-MS/MS[1]. As shown in Table 1, not all sample lots were available for analysis by LC-MS/MS as they were consumed in other testing. Smokeless tobacco cartridges were extracted using 100 mM ammonium acetate and analyzed for tobacco specific nitrosoamines (TSNAs). The four major TSNAs include: N-nitrosonicotine (NNN), N-nitrosoanabasine (NAB), N-nitrosoanatabine (NAT) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). While the published method is quite sensitive for the TSNAs (i.e. LOQ's of approximately 40 pg/mL or 40 parts per trillion), it should be noted that the extraction method used in that paper was for either finely ground tobacco or smoke captured on glass fiber filter pads . The matrix present in smokeless E-cigarette or NJoy cartridges is a spiked propylene glycol matrix supported on a fibrous material contained in a plastic housing (i.e. the inhaler component) as shown in Figure 1. The assumption was made that recovery of TSNAs from the E-cigarette cartridge assembly was as good as that published by Wu, et al., and quantitative.

For the extraction, the cartridge was removed from the inhaler unit/atomization chamber. The fibrous material was removed from the cartridge using a pair of tweezers and both the fibrous material and the white plastic housing were placed in an Ehrlenmeyer flask. The flask was weighed and the weight of the fibrous material and white plastic housing were recorded. 10 mL of 100 mM ammonium acetate and 100 µL of internal standard solution were added to the flask and the contents mixed on a flat-bed shaker for 30 minutes at ambient temperature. An aliquot of the solution was analyzed by HPLC-MS/MS.

TSNAs in the extract were quantified using deuterated internal standards. Two molecular reaction mechanisms (i.e. MRM's) were recorded for each TSNA: one for qualification and both for quantification. MRM's for each TSNA are shown in table below. The sum of the intensities of both molecular transitions was used to quantify each TSNA. TSNA content is reported as weight of TSNA per weight of nicotine/flavorant cartridge (ng/g).

MRM transitions for TSNAs

TSNA	Primary MRM	Qualifier MRM
NAB	192.1 to 162.1	192.1 to 133.1
NAT	190.1 to 160.2	190.1 to 106.1
NNK	208.1 to 122.1	208.1 to 106.0
NNN	178.1 to 148.1	178.1 to 120.1

[1] Wu, J., Joza, P., Sharifi, M., Rickett, W. Lauterbach, J. *Quantitative Method for the Analysis of Tobacco-Specific Nitrosamines in Cigarette Tobacco and Mainstream Cigarette Smoke by Use of Isotope Dilution Liquid Chromatography Tandem Mass Spectrometry*. Anal. Chem., 80, 2008, 1341-1345.

Whole Cartridges: Tobacco Specific Impurity Analysis by GC-MS and GC-MS/MS

An aliquot of each methanol extract prepared in the Detection and Quantitation of Nicotine by HPLC-UV analysis was transferred to a vial for GC-MS analysis. Samples were initially screened on an Agilent 6890 with a 5975 MSD operated in full scan mode using the method published by Man et al (Reference 3). Samples containing peaks of interest were then analyzed on a Varian 320 Triple Quad GC-MS in either single reaction monitoring mode (SRM) or multiple reaction monitoring mode (MRM) due to their increased sensitivity and selectivity. An Extracted Ion Chromatogram (EIC) looking for each impurity was obtained by individually extracting a main fragment ion for that impurity from the full scan chromatogram. Peak spectra were then compared to spectra from the NIST 05 Mass Spectral Library. All samples containing impurity peaks with spectral library matches to cotinine, anabasine, myosmine, and β-nicotyrine were than analyzed on a Varian 320 Triple Quad GC-MS in either single reaction monitoring mode (SRM) or multiple reaction monitoring mode (MRM). Identifications were based on retention time and mass spectra comparison to external standards.

Whole Cartridges: Diethylene Glycol

An aliquot of each methanol extract prepared in the Detection and Quantitation of Nicotine by HPLC-UV analysis was transferred to a vial for GC-MS analysis. Samples were screened on an Agilent 6890 with a 5975 MSD operated initially in full scan mode and later in SIM mode using the chromatographic parameters from a USP monograph procedure (Reference 5). Quantitation was performed using an external standard. The presence of diethylene glycol was confirmed in this sample by proton nuclear magnetic resonance spectroscopy by dissolving 10mg of the cartridge liquid in 500mg of D_2O and taking a spectrum on a 500 MHz NMR.

Simulated Use: Nicotine and Impurity Analysis by HSGC-MS

The cartridge contains a fiber plug within a cup. Both cup and fiber plug were placed in the same headspace vial for analysis. Compounds were identified by NIST library with greater than 90% match. Instrument parameters are detailed below.

CombiPal Headspace autosampler parameters:
Incubation: 60˚C for 15 minutes, syringe: 2000mcL gas aliquot at 145˚C,
Agitation: 250RPM, syringe fill and injection speed: 100mcL/s

Agilent 6890 Gas Chromatograph parameters (Reference 4):
Column: HP-5MS (5% diphenyl, 95%dimethyl), 15m, 0.25mm ID, 0.25 micron thickness
Temperature program: 40˚C hold 3 min, 6˚C/min to 300˚C hold 3 min, runtime: 50 minutes
Splitless injection, injector port at 280˚C, carrier gas: helium at 0.5mL/min

Agilent 5975B inert XL EI/CI MSD parameters:
Solvent delay: 1.2 min. voltage: 1705.9, low mass: 25.0, high mass: 350.0
MS quad at 150˚C, MS source at 230˚C, scan mode

Simulated Use: Sparging Apparatus: Determination of nicotine/100mL puff: trapping of nicotine and related compounds released from activation of e-cigarette

Trapping device consisted of a 150 ml gas washing bottle with sparger (see photo below). A magnetic stirring bar was added to the gas washing bottle along with 50 ml of extraction solution. Extraction solution was prepared by mixing 100 ml of acetonitrile, 11.5 g of phosphoric acid, and 800 ml of water. A draeger 100 cc hand pump was connected to the outlet of the gas washing bottle. The e-cigarette device with the selected cartridge type was connected to the inlet to the gas washing bottle via tygon tubing. The e-cigarette was butted directly up to the glass to avoid absorption. The magnet stirrer was turned on. At one minute intervals 100 cc of air were drawn through the e-cigarette into the gas washing bottle. The e-cigarette was observed to assure the LED lit indicating that the flow rate was sufficient to activate the heater in the e-cigarette. After an appropriate number of 100 cc puffs were trapped in the gas washing solution, the sample was allowed to mix for 10 minutes and the glass tubing in the gas washing bottle was rinsed with the trapping solution back into the gas washing bottle. The solution was mixed again and then sampled for analysis.