Biomarkers of exposure specific to e-vapor products based on stable-isotope labelled ingredients – methods

Landmesser A. (1); Pluym N. (1); Scherer G. (1); Sarkar M. (2), Edmiston J. (2), Scherer M. (1)

(1) Analytisch-Biologisches Forschungslabor (ABF) GmbH, Semmelweisstraße 5, Planegg, Germany

(2) Altria Client Services, Center for Research and Technology, Richmond, VA, USA

Introduction

E-vapor products (EVPs) are becoming an accepted alternative for conventional cigarettes. The popularity of EVPs is increasing and they are presently subject of a public health debate. More systematic research is required in order to assess the impact of exposure to vapor components, including potential toxicants.

The e-liquid constituents propylene glycol (PG), glycerol (G) and to some extent also nicotine (Nic) can be taken up from various sources. Therefore, EVP use-related uptake is difficult to distinguish from other sources. To overcome this problem, stable-isotope tracers are used which is the "gold" standard method in mass spectrometry for understanding kinetics, uptake and distribution of various compounds in living organisms. In the current study the e-liquid was partially replaced (10%) with stable isotope labelled $^{13}C_3$-PG, $^{13}C_3$-G and Nic-d_7. By measuring known biomarkers, this approach allows the quantitative assessment of the absorption, metabolism and further fate of PG, G and Nic as well as compounds formed from the precursors in the vapor (or endogenously from the absorbed labelled precursors) such as acrolein (ACR), propylene oxide (PO) and glycidol. To determine the labelled ingredients and their metabolites as well as their thermal degradation products methods established at ABF were adapted and in addition new methods were developed.

Correction of isotopic overlap

There are a number of naturally occurring isotopes (^{13}C, ^{15}N, ^{18}O, ^{34}S) which interfere with the analyses of the labelled biomarkers. Therefore a correction of the isotopic overlap was necessary. Knowing the structure of the measured fragment allows the calculation of the isotopic distribution of each fragment using the area ratios (area of the analyte divided by area of the internal standard):

$$L_{corrected} = L - U * P$$

$L_{corrected}$: corrected area ratio of the labelled fragment

L: area ratio of the labelled fragment

U: area ratio of the unlabelled fragment

P: calculated percentage of the isotopic distribution

Development of an LC-MS/MS method for the simultaneous determination of propylene glycol and glycerol in various matrices

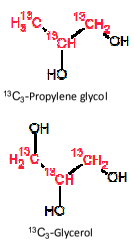

Figure 1: Structures of labelled PG and G

Propylene glycol and glycerol are the main ingredients of the e-liquids which are used in the EVPs. The percentages of PG and G in the e-liquid vary depending on the manufacturer and flavor or on the customer´s taste (in 'mix-your-own' e-liquids).

Apart from its widespread exogenous use, G is an important endogenous metabolite. In contrast, PG is an exogenous substance which has a broad range of usage in industry, cosmetics and food. Ten percent $^{13}C_3$-labelled G and PG in e-liquid were applied in the present study (Figure 1).

The challenge of the analytical method was the simultaneous determination of G and PG as well as their labelled analogues in a large range of different concentrations in various matrices.

In the literature, for each of the two substances separate methods are published using different derivatization steps and different separation techniques. A derivatisation and extraction using the Schotten-Baumann-reaction with benzoyl chloride was applied for the simultaneous determination of both analytes. The method details are summarized in the following section.

Sample preparation

- Urine/plasma/saliva samples spiked with internal standard (d_5-G, d_6-PG)
- Derivatization with benzoyl chloride
- Extraction of the derivatized analytes with pentane
- Evaporation of the organic phase
- Reconstitution of residue in acetonitrile

Analytical method

- LC-MS/MS: Sciex API 4000 (MS); Agilent 1100 (HPLC)
- Chromatographic separation (Figure 2):
  Phenomenex Kinetex EVO C18 (150 x 2.1 mm; 5 µm)
  Injection volume: 10 µL
  Eluents: acetonitrile (A) - water with 0.1% formic acid (B); flow rate: 0.4 mL/min
  Gradient: 0-0.6 min: 50% B; 0.7-2 min: 40% B; 2.1-4 min: 20% B; 4.1-6 min: 5% B; 6.1-8 min: 50% B

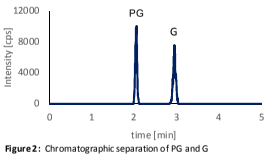

Figure 2: Chromatographic separation of PG and G

Table 1: MS parameters (IS: internal standard, RT: retention time, Quan: Quantifier, Qual: Qualifier) for propylene glycol and glycerol

Analyte	MRM [m/z]	IS (MRM) [m/z]	RT [min]	LLOQ [µg/ml]
$^{13}C_3$-PG	288 → 163 (Quan) 288 → 105 (Qual)	Propylene glycol-d_6 (291 → 169)	2.1	0.10
$^{13}C_3$-Glycerol	408 → 286 (Quan) 408 → 105 (Qual)	Glycerol-d_5 (410 → 288)	2.9	0.05

Adaption of an existing LC-MS/MS method to the analysis of deuterated nicotine and 10 of its major (deuterated) metabolites

The uptake of nicotine upon vaping depends on the amounts in the e-liquids as well as on the vaping pattern of the user. To clearly assign the nicotine uptake to vaping, the e-liquid was spiked with deuterated nicotine. The metabolism of deuterated nicotine is summarized in Figure 3.

The method is based on a published method [1], which was modified and is shortly described in the following.

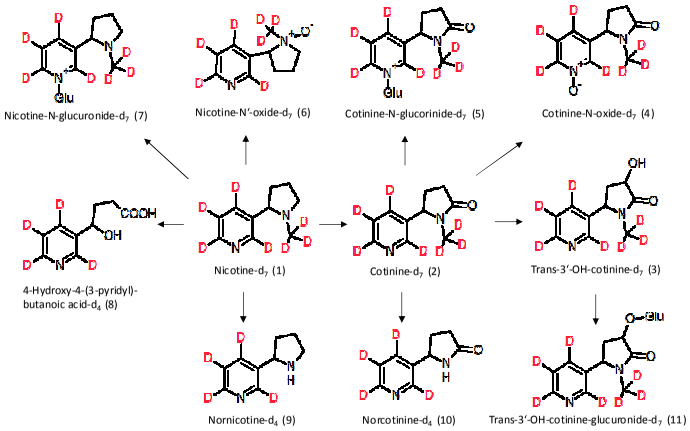

Figure 3: Metabolism of labelled nicotine to its major urinary metabolites according to [1]

Sample preparation

- Urine sample spiked with internal standard mixture (Nicotine-d_3, Cotinine-d_3, OH-Cotinine-d_3, Nicotine-N-oxide-d_3, Cotinine-N-oxide-d_3, Nicotine glucuronide-d_3, Cotinine-glucuronide-d_3, OH-Cotinine-glucuronide-d_3, Hydroxy-pyridyl butanoic acid-d_3)
- Solid-phase extraction using Oasis MCX (Waters)
- Evaporation of the organic phase
- Reconstitution of the residue in methanol

Analytical method

- LC-MS/MS: Sciex 6500⁺Qtrap (MS); Shimadzu Nexera X2 (HPLC)
- Chromatographic separation (Figure 4):
  Phenomenex Luna C8 (150 x 4.6 mm; 3 µm)
  Injection volume: 10 µL
  Eluents: methanol (A) - 0,1% ammonium acetate buffer (B); Flow rate: 0.7 mL/min
  Gradient: 0-1.0 min: 95% B; 1.0-6.0 min: 95-5% B; 6.1-8.0 min: 0% B; 8.1-11.1 min: 95% B

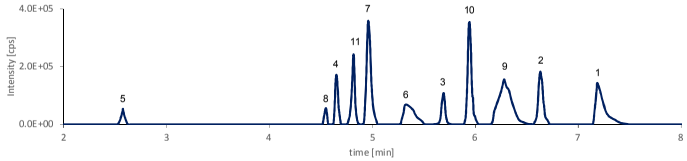

Figure 4: Chromatographic seperation of nicotine and its major labelled urinary metabolites.

Table 2: MS parameters (IS: internal standard, RT: retention time) for nicotine its major labelled urinary metabolites

Analyte (peak # in Figure 4)	MRM [m/z]	IS (MRM) [m/z]	RT [min]
Nicotine-d_7 (1)	170 → 135	Nicotine-d_3 (166 → 130)	7.2
Cotinine-d_7 (2)	184 → 84	Cotinine-d_3 (180 → 80)	6.6
Trans-3'-OH-cotinine-d_7 (3)	200 → 84	Trans-3'-OH-cotinine-d_3 (196 → 80)	5.7
Cotinine-N-oxide-d_7 (4)	200 → 102	Cotinine-N-oxide-d_3 (196 → 96)	4.7
Cotinine-N-glucorinide-d_7 (5)	360 → 184	Cotinine-N-glucorinide-d_3 (356 → 180)	2.6
Nicotine-N'-oxide-d_7 (6)	186 → 133	Nicotine-N'-oxide-d_3 (182 → 130)	5.4
Nicotine-N-glucuronide-d_7 (7)	346 → 170	Nicotine-N-glucuronide-d_3 (342 → 166)	5.0
4-Hydroxy-4-(3-pyridyl)-butanoic acid-d_4 (8)	186 → 112	4-Hydroxy-4-(3-pyridyl)-butanoic acid-d_3 (185 → 109)	4.5
Nornicotine-d_4 (9)	153 → 134	Nicotine-d_3 (166 → 130)	6.3
Norcotinine-d_4 (10)	167 → 84	Cotinine-d_3 (180 → 80)	5.9
Trans-3'-OH-cotinine-glucuronide-d_7 (11)	376 → 200	Trans-3'-OH-cotinine-glucuronide-d_3 (372 → 196)	4.8

Adaption of an existing LC-MS/MS method to the analysis of $^{13}C_3$-labelled mercapturic acids (3-HPMA, 2-HPMA, DHPMA, HMPMA, HEMA)

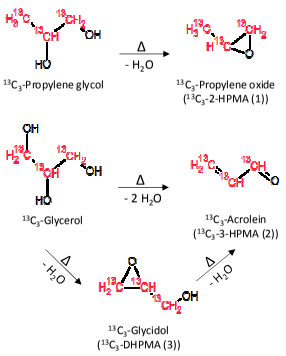

Figure 5: Selected thermal degradation products of PG and G according to [2]. The corresponding MAs are given in brackets.

Propylene oxide, acrolein and glycidol as well as ethylene oxide and crotonaldehyde are of particular interest due to their possible pyrolytic formation from G and PG according to Figure 5. Acrolein is part of the gas phase of the cigarette smoke and part of the daily diet as well as endogenous formation, which results in high background levels. Exposure to propylene oxide, glycidol, and crotonaldehyde originates mostly from cigarette smoke. To distinguish between the background and the smoke related uptake $^{13}C_3$-labelled G and PG were used for the conducted study. The aforementioned toxicants are detoxified mainly by formation and urinary excretion of mercapturic acids (MAs). With the precursors in Figures 5 and 7, the following MAs are to be expected: Acrolein: *N*-Acetyl-*S*-(3-hydroxy-$^{13}C_3$-propyl)cysteine ($^{13}C_3$-3-HPMA); propylene oxide: *N*-Acetyl-*S*-(2-hydroxy-$^{13}C_3$-propyl)cysteine ($^{13}C_3$-2-HPMA); glycidol: *N*-Acetyl-*S*-(2,3-hydroxy-$^{13}C_3$-propyl)cysteine ($^{13}C_3$-DHPMA); crotonaldehyde: *N*-Acetyl-*S*-(3-hydroxy-$^{13}C_3$-propyl-1-methyl)-L-cysteine ($^{13}C_{2/4}$-HMPMA); ethylene oxide: *N*-Acetyl-*S*-(2-hydroxy-$^{13}C_2$-ethyl)-L-cysteine ($^{13}C_2$-HEMA). The adaption of the published methods [3] are shortly described:

Sample preparation (MA Method 1)

- Urine sample spiked with internal standard (2-HPMA-d_3, ^{15}N, $^{13}C_3$-3-HPMA, DHBMA-d_7)
- Evaporation to dryness
- Reconstitution of residue in methanol

Analytical method (MA Method 1)

- LC-MS/MS: Sciex 6500⁺Qtrap (MS); Shimadzu Nexera X2 (HPLC)
- Chromatographic separation (Figure 6):
  Waters Acquity UPLC BEH HILIC (150 x 3 mm; 1.7 µm)
  Injection volume: 2 µL
  Eluents: ammonium acetate buffer (5mM) (A) – acetonitrile with 5 % ammonium acetate buffer (100 mM) (B)
  Gradient: 0-0.5 min: 1% A; 14 min: 1-7% A; 14.01-16 min: 50% A; 16.01-21 min: 95% A ; Flow rate: 0.7 mL/min

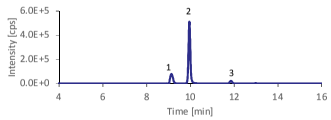

Figure 6: Chromatographic seperation of $^{13}C_3$ 2-HPMA (1), $^{13}C_3$-3-HPMA (2) and $^{13}C_3$-DHPMA (3)

Table 3: MS parameters (IS: internal standard, RT: retentiontime) for 13C3-2-HPMA, $^{13}C_3$-3-HPMA, $^{13}C_3$-DHPMA.

Analyte	MRM [m/z]	IS (MRM) [m/z]	RT [min]
$^{13}C_3$-2-HPMA	223 → 94	2-HPMA-d_3 (223 → 91)	9.2
$^{13}C_3$-3-HPMA	223 → 94	^{15}N, $^{13}C_3$-3-HPMA (224 → 91)	10.0
$^{13}C_3$-DHPMA	239 → 110	DHBMA-d_7 (257 → 128)	11.9

Sample preparation (MA Method 2)

- Urine sample spiked with internal standard (HEMA-d_3, HMPMA-d_3)
- Acidic hydrolysis
- Samples buffered (pH = 2.5)

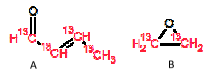

Figure 7: Structure of ^{13}C-labelled croton-aldehyde (A) and ethylene oxide (B)

Analytical method (MA Method 2)

- LC-MS/MS: Sciex API 5000 (MS); Agilent 1200 and 1100 (HPLC)
- Chromatographic separation:
  RAM-Phase: LiChrospher RP-8 ADS (25 x 4 mm; 25 µm)
  Phenomenex Luna C8 (150 x 4.6 mm; 3 µm)
  Injection volume: 50 µL
  Eluents: A & C: water with 0,1% formic acid, B: acetonitrile with 0.1% formic acid; D: 40% Eluent A; 60% Eluent B
  Gradient: 0-1.3 min: 88% A; 1.3-6 min: 88-82% A; 6-10 min: 82-52% A; 10-14 min: 52-5% A; 14-15 min: 5% A; 15-16.5 min: 0% A 10-14 min: 16.5-18 min: 88% A; RAM-Phase: 0-5 min: 100% C; 7-10 min: 0% C; 12-18 min: 100% C
  Flow rate: 0.7 mL/min

Summary and conclusion

- An analytical method for unlabeled and labeled PG and G in biological fluids was developed.
- Existing methods were successfully adapted for the analysis of deuterated nicotine and its 10 major metabolites (Nic+10) and for the analysis of the $^{13}C_3$-labelled mercapturic acids in urine.
- Correction of isotopic overlap was applied.
- The developed methods allow the specific assignment of the biomarkers to the use of EVPs.

References:

[1] Piller, M., *et al.* Simple, fast and sensitive LC–MS/MS analysis for the simultaneous quantification of nicotine and 10 of its major metabolites, *Journal of Chromatography* B. **2014**

[2] Sleiman, M., *et al.* Emissions from Electronic Cigarettes: Key Parameters Affecting the Release of Harmful Chemicals. *Environ.Sci. Technol.* **2016**

[3] Pluym, N., *et al.* Analysis of 18 urinary mercapturic acids by two high-throughput multiplex-LC-MS/MSmethods. *Anal BioanalChem*. **2015**