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# Capture of the volatile carbonyl metabolite of flecainide on 2,4-dinitrophenylhydrazine cartridge for quantitation by stable-isotope dilution mass spectrometry coupled with chromatography

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ABSTRACT

Carbonyl compounds are common byproducts of many metabolic processes. These volatile chemicals are usually derivatized before mass spectrometric analysis to enhance the sensitivity of their detections. The classically used reagent for this purpose is 2,4-dinitrophenylhydrazine (DNPH) that forms the corresponding hydrazones. When DNPH is immobilized on specific cartridges it permits solvent-free collection and simultaneous derivatization of aldehydes and ketones from gaseous samples. The utility of this approach was tested by assembling a simple apparatus for the *in vitro* generation of trifluoroacetaldehyde (TFAA) and its subsequent capture on the attached DNPH cartridge. TFAA was generated *via* cytochrome P450-catalyzed dealkylation of flecainide, an antiarrhythmic agent, in pooled human liver microsomes. Stable-isotope dilution mass spectrometry coupled with GC and LC using negative chemical ionization (NCI) and electrospray ionization (ESI) was evaluated for quantitative analyses. To eliminate isotope effects observed with the use of deuterium-labeled DNPH, we selected its $^{15}N_4$-labeled analog to synthesize the appropriate TFAA adduct, as internal standard. Quantitation by GC–NCI-MS using selected-ion monitoring outperformed LC–ESI-MS methods considering limits of detection and linearity of the assays. The microsomal metabolism of 1.5 μmol of flecainide for 1.5 h resulted in 2.6 ± 0.5 μg TFAA-DNPH, corresponding to 9.3 ± 1.7 nmol TFAA, captured by the cartridge.



## 1. Introduction

Several biochemical processes are accompanied by the formation of volatile carbonyl products. Identification and quantitation of these compounds from ambient air samples or exhaled breath have often been of interest. For example, measurement of acetaldehyde formed by the enzymatic oxidation of ethanol has been employed for metabolic flux analysis in fermentation experiments [1]. Analysis of the metabolome for volatile carbonyl compounds may also be informative for the physiological state of an individual, as well as for exposure to various drugs and environmental chemicals [2]. Notably, increased breath acetone levels are highly correlated with diabetes and have been successfully used for the noninvasive diagnosis/monitoring of diabetic patients [3]. In general, volatile carbonyls in the exhaled breath representing lipid peroxidation end-products have often been considered as potential biomarkers of oxidative stress and metabolic status [4,5]. Another important process that generates carbonyl compounds is dealkylation (heteroatom release) mediated by cytochrome P450 (CYP) during drug metabolism [6]. CYP enzymes represent the main drug metabolizing system in mammals and they also catalyze the oxidation of various endogenous (e.g., bile acids, steroids, and cholesterol) and exogenous (e.g., drugs, pollutants, and dietary components) chemicals. Since oxidative dealkylation of a drug containing an ether, thioether or alkylamino functional group produces the corresponding aldehyde in addition to the dealkylated drug (Fig. 1), measurement of an exhaled carbonyl compound may allow for noninvasive assessing of *in vivo* drug metabolism [7,8]. Due to their high volatility and reactive nature, determination of carbonyl metabolites is usually performed after derivatization to fix their concentration at a given time and/or to afford improved detection [9]. The classically used derivatization method to detect carbonyls has been the use of 2,4-dinitrophenylhydrazine (DNPH) to form the corresponding hydrazones.

Sampling of aldehydes or ketones from vapors requires impingers or bubblers [10], and the usual sample preparation procedure involves extraction with large amount of organic solvent that needs to be removed before analysis [9]. To overcome these

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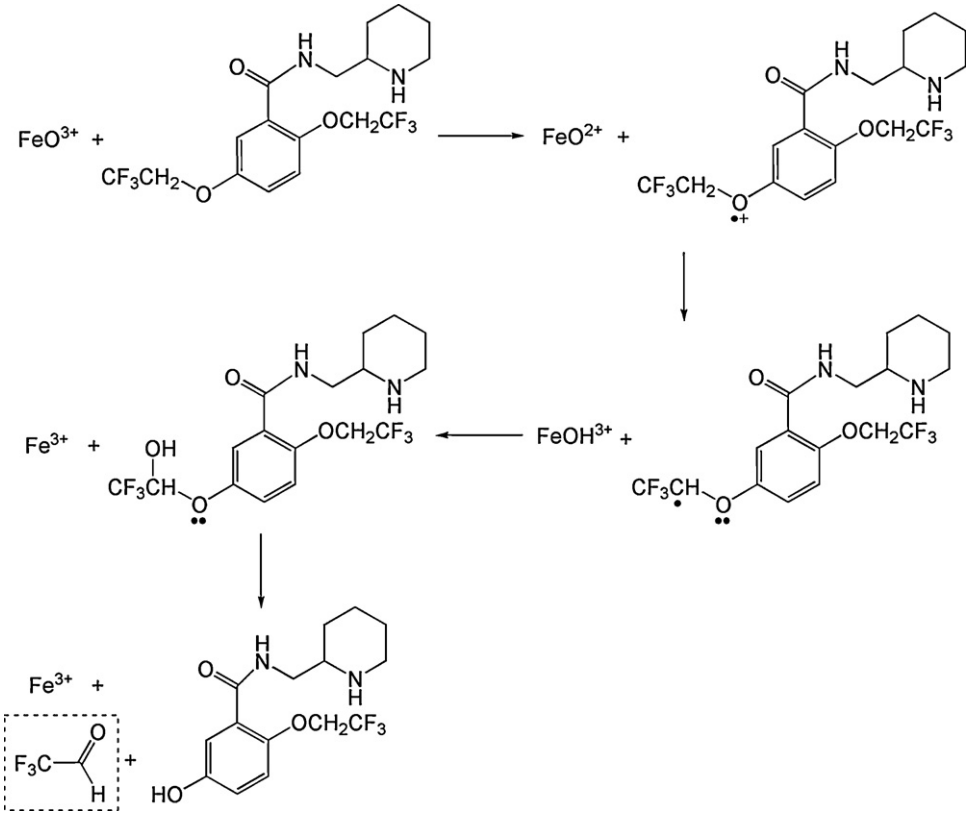


**Fig. 1.** Flecainide dealkylation by cytochrome P450 [6].

limitations, a method to capture volatile carbonyl compounds by DNPH-coated cartridges has been developed that allows solvent-free collections and simultaneous derivatization of the carbonyl compound(s) of interest [11]. Identification of the generated hydrazones is usually done by gas chromatography (GC) using flame ionization [12] or electron capture [13,14] and mass spectrometric (MS) detection [15]. With the latter, negative-ion detection has been found to be the most advantageous [9,16]. In particular, GC–MS with negative-chemical ionization (NCI) has been useful due to its high selectivity and sensitivity [17]. In addition, HPLC alone [18,19] or coupled with MS, or tandem MS (MS/MS) [9,16,20] have been utilized for carbonyl determination. In any case, an internal standard (IS) is required for accurate quantitative analysis of these volatile compounds. Although deuterium labeled hydrazones have been considered as ISs in the DNPH derivatization strategy [9], a typical obstacle is that the labeled analytes are not always available commercially or can be easily obtained by in-house synthesis. To overcome these limitations, deuterium labeled DNPH ($d_3$-DNPH) has been used to synthesize the corresponding $d_3$-labeled IS suitable for quantitation by isotope-dilution mass spectrometry [21]. An isotope effect resulting in shorter retention time for the deuterated hydrazones compared to that of corresponding unlabeled counterparts has, however, been observed upon LC or GC separation [22–24]. It has also been shown that such a deuterium isotope effect may be significant enough to change the analyte to IS peak area ratios and, therefore, to influence the accuracy of quantitations [23,25]. To eliminate possible isotope effects brought about by the use of deuterium labeled ISs, we chose $^{15}N_4$-labeled DNPH for synthesizing appropriate ISs for quantitative measurements of carbonyl compounds.

The utility of this approach was tested by assembling a simple apparatus to generate and capture trifluoroacetaldehyde (TFAA) formed *in vitro* by CYP-catalyzed dealkylation of an antiarrhythmic agent, flecainide (N-(2-piperidylmethyl)-2,5-bis(2,2,2-trifluoroethoxy)benzamide monoacetate), in pooled human liver microsomes.

## 2. Experimental

### 2.1. Chemicals

Flecainide acetate was purchased from Tocris (Ellisville, MO, USA). Pooled human liver microsomes and NADPH regenerating system were obtained from Gentest (Woburn, MA, USA) and stored at −80 °C. DNPH-coated LpDNPH S10 cartridges were purchased from Supelco (Bellefonte, PA, USA). Microcentrifuge tubes (2 mL) and hypodermic needles (20G × 1½ in.) were supplied by USA Scientific (Ocala, FL, USA) and Air-Tite Products Co., Inc. (Virginia Beach, VA, USA), respectively. Solvents were of analytical grade and obtained from Fisher Scientific (Atlanta, GA, USA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). Preparative thin layer chromatography (TLC) plates (Silica Gel G 20 cm × 20 cm scored, UNIPLATE-T Taper Plate) were purchased from Analtech (Newark, DE, USA).

### 2.2. Apparatus to generate and capture volatile carbonyl metabolites

Fig. 2 shows the volatile carbonyl-trapping apparatus we assembled in house and used in this study. An LpDNPH S10 cartridge (B) was connected to a 2-mL polypropylene microcentrifuge tube (A) by a hypodermic needle punctured through the closed top to collect the aldehyde released into the headspace. This hypodermic needle was carefully positioned, so that no solution from the incubated mixture could enter into the connected cartridge. A vacuum pump (KNF Neuberger Inc., Trenton, NJ, USA) was connected to the open

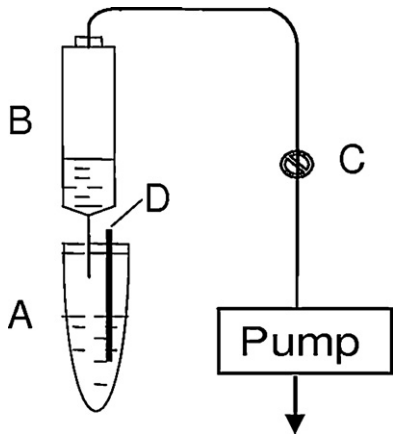


**Fig. 2.** Schematic illustration of the aldehyde-generating and trapping apparatus. (A) Microsomal incubation mixture; (B) LpDNPH cartridge; (C) two-way valve; and (D) hypodermic needle (for aspiration of the system and agitation of the mixture).

end of the cartridge to provide a continuous gentle suction. Another hypodermic needle (D) punctured through the top to reach the bottom of the closed centrifuge tube was used to aspirate the system, agitate the incubation mixture and purge out the generated volatile aldehyde from the solution phase *via* continuous bubbling. A two-way valve (C) was used to adjust the vacuum to obtain about 1 bubble/s at the end of the needle (D) submerging into the solution giving thereby an estimated purge flow rate of 0.7–0.8 mL/min.

### 2.3. Synthesis of TFAA-DNPH and its labeled analogs as an internal standard for isotope-dilution mass spectrometry

The deuterium labeled DNPH ($d_3$-DNPH) was synthesized as described before [21]. $^{15}N_4$-DNPH was prepared from dinitro[$^{15}N_2$]-chlorobenzene and $^{15}N_2$-hydrazine sulfate in a similar manner [26]. To obtain an authentic TFAA-DNPH synthetic standard, trifluoroacetaldehyde ethyl hemiacetal (310 μL) was dissolved in 15 mL toluene followed by addition of 480 mg DNPH and 15 mg *p*-toluenesulfonic acid. Molecular sieve 4A (1 g) was then added, and the mixture was stirred for 4 h at 100 °C. After addition of 10 mL diethyl ether, the solution was extracted with 1% (w/v) sodium bicarbonate. The organic layer was then separated, washed with brine and dried over sodium sulfate. The solvent was evaporated under reduced pressure resulting in a yellow solid. The product was purified on preparative TLC using hexane:ethyl acetate = 6:1 (v/v), $R_f$ = 0.63. Trifluoroacetaldehyde [$^{15}N_4$]-2,4-dinitrophenylhydrazone (TFAA-$^{15}N_4$-DNPH) as well as trifluoroacetaldehyde 2,4-dinitro-3,5,6-trideuterophenylhydrazone (TFAA-$d_3$-DNPH) were obtained analogously using the corresponding labeled DNPHs.

### 2.4. In vitro generation of TFAA via microsomal incubation

A microsomal incubation was performed at 37 °C in a suspension containing 2 mg/mL human liver microsomes, an NADPH-generating system consisting of 5.2 mM $NADP^+$, 13.2 mM glucose-6-phosphate, 1.6 U/mL glucose-6-phosphate dehydrogenase, 13.2 mM $MgCl_2$ and 1 μmol/mL flecainide in 100 mM potassium phosphate buffer (pH 7.5). The total volume of the incubation mixture was 1.5 mL. The metabolically formed TFAA was collected for 1.5 h by the apparatus shown in Fig. 2. After sample collection, 0.8 μg of TFAA-$^{15}N_4$-DNPH IS (40 μg/mL in acetonitrile) was added to the cartridge that was subsequently washed with 3× 1 mL acetonitrile. The solvent was then removed from the collected solution at room temperature under a nitrogen stream, and the residue was dissolved in the appropriate solvent (100 μL) for analyses.

### 2.5. GC–NCI-MS analysis

A PolarisQ mass spectrometer system interfaced to a TRACE GC and controlled by Xcalibur 1.4 data system (all from Thermo Electron Corporation, Trace Chemical Analysis, Austin, TX, USA) was used in the study. Separations were done on a 30 m × 0.25 mm i.d. Rtx-5MS ($d_f$ = 0.25 μm) fused silica column (Restec, Bellefonte, PA, USA). The injector temperature was 220 °C. The carrier gas was helium at a flow rate of 1.0 mL/min. Column head pressure was 7.8 psi. All injections (1 μL volume) were carried out using splitless mode. The oven temperature was kept at 40 °C for 1 min, then increased to 300 °C at 25 °C/min and maintained at 300 °C for 3 min. Conditions for mass spectrometry were as follows: ion source temperature, 200 °C; interface temperature, 300 °C; ionizing voltage, 70 eV; NCI mode with methane as a reagent gas. TFAA-DNPH, TFAA-$d_3$-DNPH, and TFAA-$^{15}N_4$-DNPH were detected at 182, 185, and 185 *m/z*, respectively.

### 2.6. LC–ESI-MS analysis

Online LC–MS analysis was performed using a LCQ 3D ion-trap instrument equipped with electrospray ionization (ESI) source operated in negative mode and coupled with a P-1000 HPLC pump controlled by Xcalibur 1.3 data system (Thermo Electron Corporation, Trace Chemical Analysis, Austin, TX, USA). Separations were done on an Ascentis Express fused-core C18 column (5 cm × 2.1 mm i.d., 2.7 μm; Supelco, Bellefonte, PA, USA). Isocratic elution was performed with 52% acetonitrile at a flow rate of 0.2 mL/min. Samples were dissolved in the mobile phase and the injection volume was 5 μL. The study was carried out at room temperature. ESI spray voltage and capillary temperature were maintained at 4.5 kV and 200 °C, respectively. Scans were performed in the range of *m/z* 275–283, the extracted ions of *m/z* 277, 280, and 281 were used for the quantitation of TFAA-DNPH, TFAA-$d_3$-DNPH, and TFAA-$^{15}N_4$-DNPH, respectively. The same ions were monitored during selected ion monitoring (SIM) analysis. For MS/MS experiments collision-induced dissociation (CID) was performed using 1.0-u isolation width and 30% normalized collision energy with helium as the collision gas. The selected reaction monitoring (SRM) transition was *m/z* 277 → 179 for TFAA-DNPH.

### 2.7. Isotope effects using stable isotope labeled TFAA-DNPH adducts

The percentile single isotope effects (%IEs) of TFAA-$d_3$-DNPH and TFAA-$^{15}N_4$-DNPH were calculated according to the equation given by Turowski et al. [27]. The samples containing both the IS and the analyte were dissolved in acetonitrile for GC–MS and in the mobile phase for LC–MS analyses, respectively. The GC and LC chromatographic parameters (oven temperature and mobile phase composition, respectively) were set to achieve similar $k'$ (retention factor) values for both chromatographic techniques. Accordingly, isothermal GC analyses (with 1:50 split injection) were performed at 158 °C, 173 °C, 190 °C, and 203 °C oven temperature, and LC elutions were done with 40%, 45%, 50%, and 55% acetonitrile, respectively. The injected quantity of analyte and IS were sufficiently small (1.0 ng and 5.0 ng) to approach infinite-dilution in the mobile phase. Other experimental parameters were identical to those described in Sections 2.5 and 2.6 for GC– and LC–MS, respectively.

### 2.8. Assay validations

GC–MS and LC–MS methods were validated in accordance with the US Food and Drug Administration (FDA) Guidance [28]. Limit of detections (LODs) were calculated according to the International Conference on Harmonisation (ICH) guidelines [29], based on the standard deviation of the *y*-intercepts and the slope of regression lines. LOD confidence intervals were determined by the method of Beránek et al. [30]. Assay calibration was done by isotope dilution using six different analyte/IS molar ratios [31]. The quantity of IS was kept constant at 50 ng injected on column for both chromatographic methods. Calibration curves were obtained using linear regression (with or without weighting) resulting in coefficient of determination ($R^2$) of higher than 0.99. The appropriate weighting scheme was selected according to the recommendations of Almeida et al. [32]. Accuracy, indicating the extent of agreement between measured ($C_M$) and nominal concentrations ($C_Q$) of the analyte (TFAA-DNPH) in the quality control (QC) samples, was estimated at various analyte concentrations with 50 ng IS and using $n = 5$ replicates. Percentage accuracy was calculated as $[(C_M - C_Q)/C_Q] \times 100$ [31–34].

TFAA generated *in vitro* by CYP-mediated microsomal metabolism (Fig. 1) and captured by the apparatus shown in Fig. 2 was identified as its DNPH derivative by comparing the corresponding retention time and NCI mass spectrum with those of the authentic synthetic reference compound. For quantitation, SIM using the most intense fragment ion of the analyte and $^{15}N_4$-DNPH-derived IS (182 and 185 *m/z*, respectively) was employed. The amount of TFAA-DNPH captured by the cartridge was calculated using triplicate experiments by multiplying the ratio of the analyte to IS peak areas of the SIM chromatograms with the known quantity (0.8 μg) of the IS added onto the LpDNPH S10 cartridge [31,33,34]; data are given as mean ± SD.

## 3. Results and discussion

### 3.1. A simple apparatus coupled with a DNPH-coated cartridge to capture in vitro formed TFAA

Volatile aldehydes and ketones are common end- and/or site-products of many biochemical processes and drug metabolisms. DNPH derivatization is perhaps the most generally utilized sample preparation method for their analyses [9]. A simplified measurement of these compounds can take advantage of the commercially available DNPH-coated cartridges to avoid cumbersome sample preparation before GC–MS or LC–MS analyses [11]. With these cartridges, volatile carbonyls are captured through their *in situ* conversion to the corresponding hydrazones that are subsequently eluted for analysis with small amount of an organic solvent, such as acetonitrile.

In the present study, the aldehyde (TFAA), formed metabolically upon microsomal incubation of flecainide (Fig. 1) was trapped by a DNPH cartridge from the headspace of a simple experimental apparatus assembled in-house (Fig. 2) for quantitative analysis. We believe that a similar device may also be considered to replace the separate vial containing DNPH solution introduced to capture acetaldehyde after diffusion through the gas headspace in metabolic flux analysis of fermentation experiments [1], or to perform metabolomic studies focusing on volatile carbonyl compounds [2].

No authentic standard gas sample is available or can be prepared to test the commercially available DNPH-coated LpDNPH S10 cartridges (Supelco) for TFAA. However, according to the manufacturer's specification, the recoveries of formaldehyde and acetaldehyde are 103–106 and 120–135%, respectively.

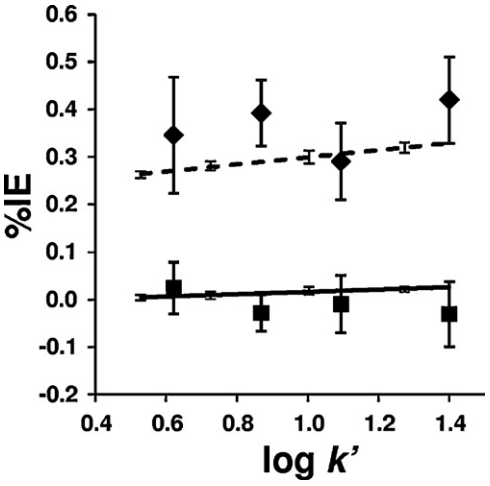


**Fig. 3.** Percentile single isotope effects (%IEs) of deuterium- and $^{15}N$-labeled TFAA-DNPHs plotted against log *k*′. HPLC (♦) and GC (no symbol) data of TFAA-$d_3$-DNPH; HPLC (■) and GC (no symbol) data of TFAA-$^{15}N_4$-DNPH. Solid line represents the linear regression fit of TFAA-$^{15}N_4$-DNPH GC data ($R^2 = 0.9641$), dashed line shows the linear regression fit of TFAA-$d_3$-DNPH GC data ($R^2 = 0.9946$).

These values indicate that the overall efficiency of collection, derivatization and elution processes are essentially quantitative for volatile aldehydes and ketones using these cartridges.

### 3.2. Selection of isotope-labeled internal standard for quantitative determination of TFAA

The %IEs of the labeled hydrazones plotted against log *k*′ are shown in Fig. 3 for LC and GC analyses. Regardless of the analyte's concentration and the chromatographic technique applied, the %IEs of deuterium and heavy-nitrogen labeled isotopes were clearly different. Notably, %IE for the TFAA-$^{15}N_4$-DNPH was practically zero under all experimental conditions. On the other hand, and in agreement with a previous report on structurally related compounds [27], the deuterated counterpart showed significant isotope effect. While an increase in %IE with decreasing organic content of the mobile phase (methanol) has been reported [27], in the present study we could not observe statistically significant change in %IE ($0.36 \pm 0.06$), when the concentration of the acetonitrile was changed (from 40 to 55% changing, thus, *k*′ from 3.4 to 25) upon LC separation. On the other hand, we have obtained very tight GC data, which was apparently because of more reproducible control of the parameters affecting separation in GC (pressure, flow rate, temperature, etc.) than in the routine LC we employed, ranging from 0.26 to 0.32 in %IE, when the effect of increasing *k*′ was investigated by isothermal GC. Moreover, the fitted trend line indicated a slight but statistically significant effect of *k*′ on %IE (a positive slope of $0.075 \pm 0.004$ of the %IE versus log *k*′ plot) using GC. Due to the isotope effect combined with the high chromatographic resolution of the technique, GC peaks of deuterium labeled and non-labeled TFAA-DNPH were even partially resolved at 158 °C oven temperature (Fig. 4A, C, and E), while no separation was observed with the $^{15}N$-labeled compound implicating the advantage of heavy-nitrogen over deuterium labeling of the IS in quantitative analyses. For simple sample matrices, the impact of isotope effects associated with deuterium labeling may be minimized at the expense of chromatographic separation by rapid temperature program in GC, or by steep mobile phase gradient when LC is used. However, these measures could have detrimental consequences on quantitation from complex sample matrices [23,25].

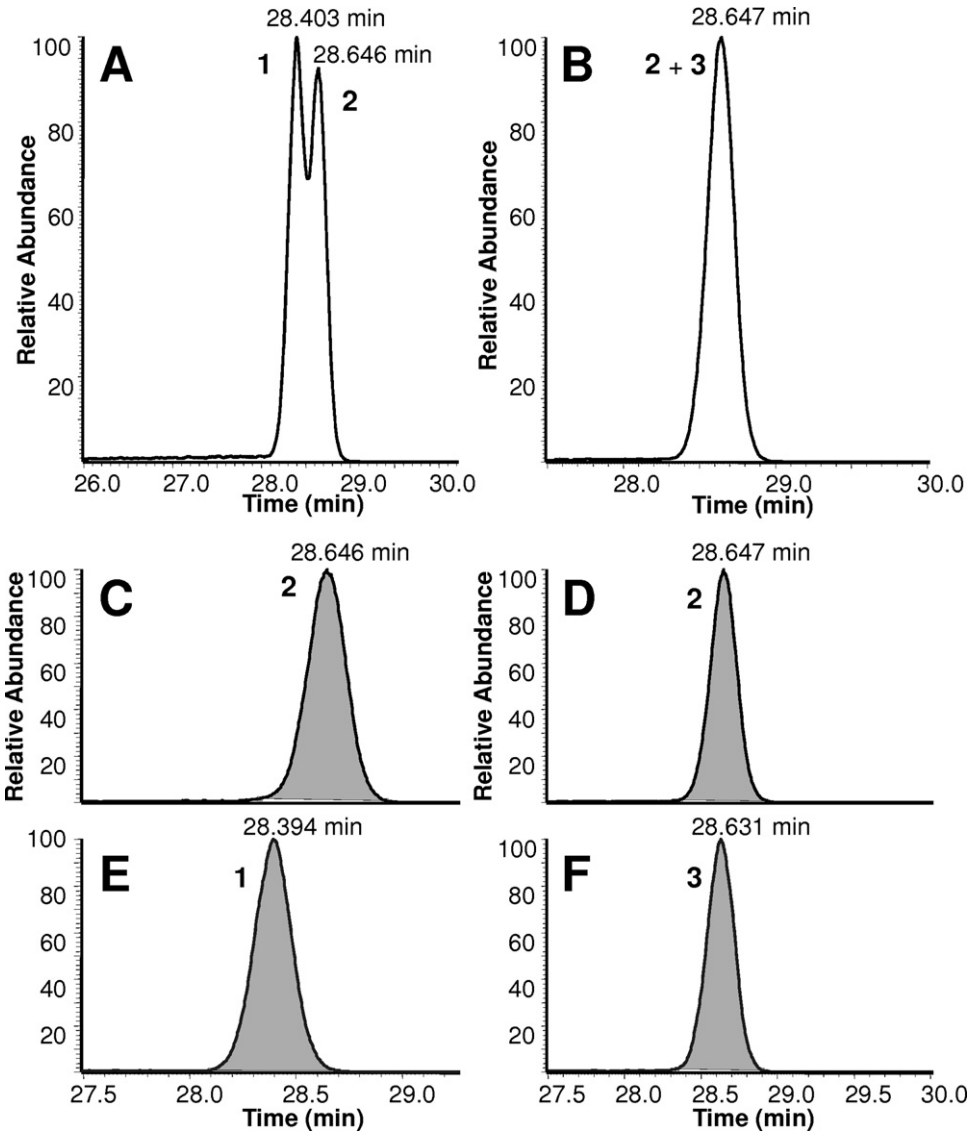


**Fig. 4.** Total ion chromatograms of the partially resolved peaks of $d_3$- and non-labeled TFAA-DNPH (A); and practical co-elution of $^{15}N_4$- and non-labeled TFAA-DNPH (B), extracted ion chromatograms of the non-labeled TFAA-DNPH (C, D; *m/z*, 182), the $d_3$-labeled (E; *m/z*, 185) and the $^{15}N_4$-labeled TFAA-DNPH (F; *m/z*, 185); (**1**) TFAA-$d_3$-DNPH, (**2**) TFAA-DNPH, and (**3**) TFAA-$^{15}N_4$-DNPH; 50 ng/μL injection, split ratio 1:50, flow rate 1 mL/min, oven temperature 158 °C.

### *3.3. Performance parameters of TFAA-DNPH quantitation by GC–MS and LC–MS*

As shown in Fig. 5, NCI-MS fragmentation between the two N atoms of the hydrazone moiety yielded the base peaks of the mass spectra of the analyte (*m/z*, 182) and IS (*m/z*, 185) and, therefore, their *m/z* values differed by 3 u. SIM-based quantitation was performed by using these two ions [17]. Fig. 6A shows the negative-ion ESI mass spectrum of TFAA-DNPH, and Fig. 6B depicts its CID–MS/MS with the origin of characteristic fragments indicated on the structure. The corresponding mass spectra of the isotope labeled compounds, TFAA-$d_3$-DNPH and TFAA-$^{15}N_4$-DNPH, are presented in Supplemental Figs. S7 and S8. An advantage of LC–MS would be the reliance on molecular ions ($[M-H]^-$, Fig. 6A) instead of fragment ions (Fig. 5) for quantitative analyses.

For quantitation, GC–NCI-MS with SIM proved to be, however, more sensitive based on LODs than LC–ESI in any modes (SIM, narrow-range scans and SRM) of data acquisition using the default parameters for the quadrupole ion trap (LCQ) and software (XCalibur 1.3) specified in Section 2.6. Specifically, we estimated $0.6 \pm 0.2$ ng/mL TFAA (as low as $1.7 \pm 0.6$ pg TFAA-DNPH per injection) of LOD for GC–MS, while LODs using LC–MS were $16 \pm 4$, $23 \pm 6$ and $59 \pm 32$ ng/mL for SIM, narrow-range scans and SRM, respectively. GC–MS also outperformed LC–MS regarding assay linearity. Therefore, we chose to focus on validating GC–NCI-SIM for quantitative analysis of TFAA (Table 1) generated from flecainide according to the experiment described in Section 2.4. Nevertheless, LC–MS assays have also passed validation criteria (Supplementary Table 1).

Table 1 summarizes the method validation results of the GC–NCI-SIM technique. To examine intra-day and inter-day precision, QC samples were analyzed on three consecutive days at three concentration levels. The repeatability (expressed as percentage of the relative standard deviation, RSD%) of either the analyte-to-IS area ratios and retention times were within the acceptable range [28] at any of the selected TFAA-DNPH quantities injected on the GC-column. Accuracy, indicating the extent of agreement between the measured ($C_M$) and nominal concentration ($C_Q$) of the analyte, was also acceptable [28] in the concentration range estimated to match the quantity of TFAA formed metabolically in the test experiment (Section 2.4). To demonstrate the stability of standard

**Table 1**
Summary of the GC–NCI-MS method validation.

| | Repeatability | | | |
|---|---|---|---|---|
| | Area ratio (RSD%)[a] | | Retention time (RSD%)[b] | |
| TFAA-DNPH (ng injected) | Intra-day[c] | Inter-day[d] | Intra-day[c] | Inter-day[d] |
| 5 | 1.1 | 5.5 | 0.02 | 0.02 |
| 10 | 6.5 | 7.1 | 0.01 | 0.01 |
| 100 | 2.1 | 3.2 | 0.02 | 0.80 |

| | QC | | |
|---|---|---|---|
| $C_Q$[e] (ng/μL) | $C_M$[f] ± SD (ng/μL) | Precision (RSD%) | Accuracy (%) |
| 5 | 5.58 ± 0.03 | 0.5 | 11.6 |
| 50 | 50.6 ± 1.6 | 3.2 | 1.2 |
| 150 | 147 ± 3 | 2.1 | −2.1 |

[a] Area ratio (RSD%) denotes precision expressed in RSD% of analyte/IS area ratios.
[b] Retention time (RSD%) denotes precision expressed in RSD% of retention times.
[c] $n = 5$.
[d] $n = 15$.
[e] $C_Q$ denotes the nominal concentration of TFAA-DNPH in QC samples.
[f] $C_M$ denotes the measured concentration of TFAA-DNPH in QC samples.

solutions, QC samples at two concentrations (5 and 50 ng/μL) were stored at room temperature and reanalyzed after 48 h. The results confirmed that peak area ratios and retention times did not change significantly.

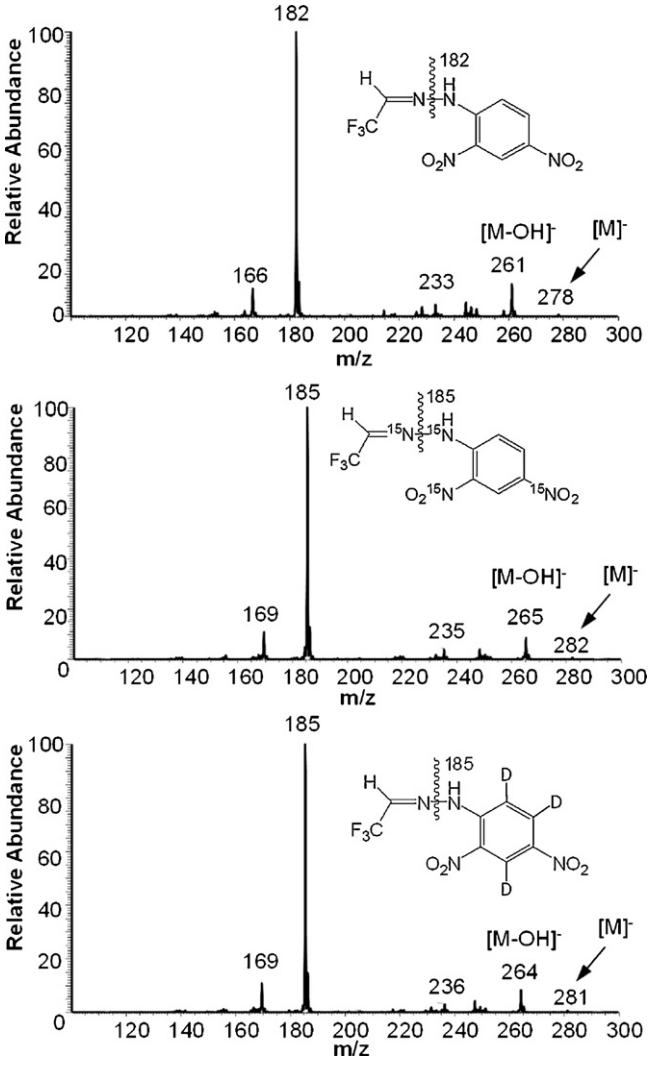


**Fig. 5.** Full-scan NCI-MS spectra of TFAA-DNPH and its $^{15}N_4$- and $d_3$-labeled analogs.

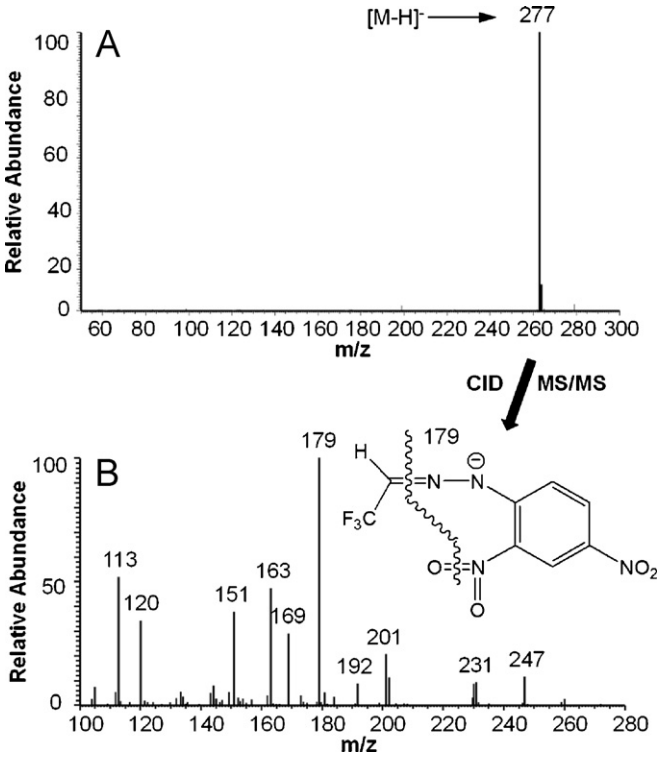


**Fig. 6.** Full-scan ESI-MS spectrum of TFAA-DNPH (A) and CID-MS/MS spectrum of the $[M-H]^-$ ion, $m/z$ 277 (B).

### 3.4. Quantitative determination of TFAA produced by microsomal metabolism of flecainide

While efficient carbonyl collection from gas samples can be achieved by employing DNPH cartridges [35], in the present study we also wished to investigate the role of $^{15}N$-labeled IS for allowing accurate quantitative analyses of the captured carbonyl derivatives by isotope-dilution mass spectrometry. We selected the *in vitro* formation of TFAA from the antiarrhythmic agent flecainide in human liver microsome as a model system for this endeavor (Fig. 1). First, for the unambiguous identification of TFAA-DNPH removed from the cartridge, an authentic synthetic sample was prepared.

Using the GC retention time and NCI-MS spectrum of the synthetic standard, we have unequivocally identified the compound

collected from the *in vitro* experiment as TFAA-DNPH (data not shown).

In order to accurately measure carbonyls after DNPH cartridges, it is important to probe the collection efficiency of the cartridge for the particular analyte. Although previous studies have shown that the cartridge's performance in humid air affords essentially quantitative capture of volatile aldehydes and ketones [35], we have tested for a potential loss of the labeled hydrazone during sample elution through exchange with the unlabeled DNPH coated on the adsorbent of the cartridge. We concluded that such exchange was not detectable. Similar observation with $d_3$-hydrazones has also been reported earlier [21].

Based on results described in Sections 3.2 and 3.3, GC–NCI-MS analysis of our target compound with $^{15}$N-stable-isotope labeled IS was chosen to determine TFAA produced during *in vitro* microsomal metabolism of flecainide (Fig. 1). When 1.5 μmol of flecainide was incubated with liver microsomes under the experimental conditions specified in the present study (Fig. 2 and Section 2.4), $2.6 \pm 0.5$ μg of TFAA-DNPH, corresponding to $9.3 \pm 1.7$ nmol of TFAA was captured from the headspace of the vial based on three independent experiments.

## 4. Conclusions

We have proposed a convenient method for the quantitative analysis of a volatile carbonyl metabolite trapped on commercially available DNPH cartridges. The application of $^{15}$N- labeled IS is more advantageous than deuterium labeling, because $^{15}$N-labeling did not induce chromatographic isotope effects, hence, it would not influence the accuracy of the quantitation in LC-MS or GC-MS analyses. The use of $^{15}N_4$-DNPH to prepare the corresponding hydrazones, as ISs, permits quantitation of captured aldehydes and ketones of metabolic origin by stable-isotope dilution. Because the breath test is becoming one of the most desirable noninvasive procedures for clinical diagnostics [36], the analytical methods presented here may also be adopted for clinical diagnosis, disease state assessment, drug monitoring and evaluation of environmental exposure, when the underlying processes are manifested by the appearance or changes in the concentration of volatile carbonyl compounds in the expired air.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.01.067.

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