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# Interconversion of ephedrine and pseudoephedrine during chemical derivatization

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**Gas chromatography–mass spectrometry (GC-MS) analysis after heptafluorobutyric anhydride (HFBA) derivatization was one of the published methods used for the quantification of ephedrine (EP) and pseudoephedrine (PE) in urine. This method allows the clear separation of the derivatized diastereoisomers on a methyl-silicone-based column. Recently the authors came across a human urine sample with apparently high levels (μg/ml) of EP and PE upon initial screening. However, duplicate analyses of this sample using the HFBA-GC-MS method revealed an unusual discrepancy in the estimated levels of EP and PE, with the area response ratios of EP/PE at around 29% on one occasion and around 57% on another. The same sample was re-analyzed for EP and PE using other techniques, including GC-MS after trimethylsilylation and ultra-high-performance liquid chromatography-tandem mass spectrometry. Surprisingly, the concentration of EP in the sample was determined to be at least two orders of magnitude less than what was observed with the HFBA-GC-MS method. A thorough investigation was then conducted, and the results showed that both substances could interconvert during HFBA derivatization. Similar diastereoisomeric conversion was also observed using other fluorinated acylating agents (e.g. pentafluoropropionic anhydride and trifluoroacetic anhydride). The extent of interconversion was correlated with the degree of fluorination of the acylating agents, with HFBA giving the highest conversion. This conversion has never been reported before. A mechanism for the interconversion was proposed. These findings indicated that fluorinated acylating agents should not be used for the unequivocal identification or quantification of EP and PE as the results obtained can be erroneous. Copyright © 2012 John Wiley & Sons, Ltd.**

**Keywords:** ephedrine; pseudoephedrine; interconversion; gas chromatography; ultra-high performance liquid chromatography; mass spectrometry

## Introduction

*Ephedrae sinica* (Ma Huang) has long been used in traditional Chinese medicine.[1,2] The main ephedra alkaloids isolated from this species, typical of sympathomimetic amines, include ephedrine (EP), pseudoephedrine (PE), methylephedrine, and other homologous substances.[2–4] As with other sympathomimetic agents, some of these alkaloids can be found in medications for the treatment of colds and influenza, nasal congestion, asthma, coughs. and bronchitis.[5] Ephedra-containing supplements are also marketed as natural aids for weight control and for boosting sports performance in a number of countries.[2] However, ephedra is known to have adverse health effects, including heart attacks, strokes, and psychosis. Deaths allegedly related to ephedra have also been reported.[6–8] For this reason, the sale of ephedra-containing supplements is banned in some countries including the USA.[9] In addition, the World Anti-Doping Agency (WADA) has prohibited some of the ephedra alkaloids.[10]

The separation and quantification of EP and PE in different biological matrices using gas chromatography (GC),[11–16] liquid chromatography (LC),[17,18] and capillary electrophoresis[19,20] have been extensively reported. In the authors' laboratory, a published method had been adopted for a few years in the analysis of EP and PE, which was based on liquid-liquid extraction and heptafluorobutyric anhydride derivatization followed by gas chromatography–mass spectrometry analysis (HFBA-GC-MS).[15] The excellent separation achieved by this method between the derivatized EP and PE has allowed an unambiguous identification of either or both diastereoisomers at low levels.

During the course of our regular drug screening, a human urine sample gave a presumptive result indicating a high concentration of EP and/or PE (Sample 1). Since the screening method applied on Sample 1 was incapable of separating EP and PE, a follow-up analysis (run in duplicate) using the aforementioned HFBA-GC-MS method[15] was performed in order to confirm the identity of the substance(s) present. Results of this follow-up analysis confirmed the presence of both EP and PE in the sample. However, a significant discrepancy on the area response ratios of EP to PE was observed between the duplicate measurements (i.e. EP/PE (replicate A) = 29 %; EP/PE (replicate B) = 57%; Table 1).

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**Table 1.** Area response ratios in Sample 1, Sample 2, and negative urine spiked with either EP or PE using different analytical methods

| Method | Sample 1 (EP/PE) | Sample 2 (EP/PE) | EP spiked sample (PE/EP) | PE spiked sample (EP/PE) |
|---|---|---|---|---|
| HFBA-GC-MS (Replicate A) | 29 % | 7 % | 8 % | 12 % |
| HFBA-GC-MS (Replicate B) | 57 % | (Not applicable) | (Not applicable) | (Not applicable) |
| PFPA-GC-MS | 3 % | 2 % | 0.8 % | 2 % |
| TFAA-GC-MS | 0.5 % | EP not detected | 0.7 % | 6 % |
| TMS-GC-MS | EP not detected | EP not detected | PE not detected | EP not detected |
| UHPLC-MS/MS | EP not detected | EP not detected | PE not detected | EP not detected |

Since isotopic labelled EP (i.e. $d_3$-EP) had been used as the internal standard, such discrepancy was considered very unusual. An ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method, was then applied to this sample with the objective to verify the above findings. Surprisingly, only PE was detected; no EP. A further test was performed on Sample 1 using GC-MS after trimethylsilylation (TMS), and the results agreed with those from UHPLC-MS/MS. These findings strongly suggested that the EP identified with the HFBA-GC-MS method was an artefact derived from the conversion of PE to EP during HFBA derivatization. This study reports a series of experiments aiming to determine the cause of the observed diastereoisomeric conversion between EP and PE. A plausible mechanism of the interconversion is also proposed.

## Materials and methods

### Materials

Ephedrine hydrochloride (99.7%) was obtained from British Pharmacopeia (London, UK), $d_3$-ephedrine ($d_3$-EP) hydrochloride (99%) was from Cerilliant Corporation (Round Rock, TX, USA), and pseudoephedrine hydrochloride was from United States Pharmacopeia (Rockville, MD, USA). Acetonitrile (LiChrosolv®), ammonium acetate (Extra Pure grade), dichloromethane (GR grade), *n*-heptane (LiChrosolv®), hydrochloric acid (30%, Suprapur), methanol (LiChrosolv®), potassium carbonate (Pro Analyse grade) and potassium hydroxide (pellets) were obtained from Merck (Darmstadt, Germany). *N,O*-bis(trimethylsilyl)fluoroacetamide (BSTFA) was purchased from Sigma-Aldrich (St Louis, MO, USA). Sodium sulfite was purchased from Peking Chemical Works (Beijing, China), and anhydrous sodium sulfate was from Farco Chemical Supplies (Beijing, China). Deionised water was generated from an in-house water purification system (Milli-Q, Molsheim, France). Heptafluorobutyric anhydride (HFBA), pentafluoropropionic anhydride (PFPA), trifluoroacetic anhydride (TFAA) and trimethylchlorosilane (TMCS) were obtained from Pierce (Rockford, IL, USA).

### Sample preparation procedures

Urine (1 ml) after centrifugation to remove particulate matter was spiked with $d_3$-EP (100 ng). Sodium sulfite (50 µl; 25 % aq., *w/v*) and potassium carbonate buffer (50 µl; 4 M) were added to the sample and the pH was adjusted to 10.0 using potassium hydroxide (0.1 M) and hydrochloric acid (0.1 M). The mixture was then extracted with dichloromethane (4 ml) and centrifuged at 1500 × *g* for 10 min. The organic layer was removed and dried using anhydrous sodium sulfate, and the solvent was evaporated to dryness under nitrogen at ambient temperature. For UHPLC-MS/MS analyses, the dried residue was reconstituted in 50 µl of 2% acetonitrile in ammonium acetate buffer (3 mM, pH 3.8, *v/v*) and injected directly. For GC-MS analyses, TMS was achieved by adding 30 µl of BSTFA (5% TMCS, *v/v*) to the dry residue and incubated at 60 °C for 30 min. Fluorinated-acylation was achieved by adding acetonitrile (100 µl) and the corresponding fluorinated-acyl anhydride (30 µl; TFAA, PFPA or HFBA) to the dry residue, mixing thoroughly and incubating at 60°C for 15 min. Each reaction mixture was then evaporated to dryness under nitrogen at ambient temperature and reconstituted in 50 µl of *n*-heptane for GC-MS analysis.

### Instrumentation

GC-MS analyses were performed on an Agilent 6890N Network GC system coupled to an Agilent 5973 Network Mass Selective Detector (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed on a DB-5MS GC column (~ 30 m × 0.25 mm, 0.25 µm film thickness) with a constant helium flow of 1.2 ml/min. The oven temperature was set initially at 80 °C for 1.0 min, increased to 200 °C at 5 °C/min and then to 320 °C at 45 °C/min, and finally held at 320 °C for 3 min. Samples (1 µl each) were injected at 250 °C in the splitless mode. All GC-MS analyses were performed in the EI mode with full-scan acquisition. The mass range acquired was from *m/z* 40 to 600.

UHPLC-MS/MS analyses were performed on a Waters Quattro Premier XE Mass Spectrometer (Waters Corporation, Milford, MA, USA) equipped with a Waters Acquity UHPLC system. A reversed-phase Waters Acquity UPLC® BEH C8 column (10 cm 2.1 mm ID; 1.7 µm particle size) was used for the separation. An isocratic program was run at 400 µl/min for 8 min ($t$ = 0–8 min) at the composition of 98% 3 mM ammonium acetate pH 3.8 buffer (solvent A) and 2% acetonitrile (solvent B). After each run, the system was purged with 100 % solvent B for 1.9 min ($t$ = 8.1–10 min). The solvent composition was then returned to the initial settings at $t$ = 10.1 min, and equilibrated until $t$ = 12 min before the next injection. The injection volume was 5 µl each. The atmospheric pressure ionization (API) source was operated in positive ESI mode with a spray voltage of + 3.5 kV. The extractor voltage and the RF lens voltage were 5 V and 0.2 V respectively. The desolvation temperature was 350 °C and the source temperature was 110 °C. The desolvation gas flow and cone gas flow were respectively 600 L/hr and 50 L/hr. The mass spectrometer was operated in the selected-reaction monitoring (SRM) mode. The dwell time for each transition was 50 msec, with 5 msec inter-channel/inter-scan delay. Cone voltage was 15 V and collision energies for all SRM transitions were 15 eV. The SRM transition chosen for monitoring both EP and PE was *m/z* 166 to *m/z* 148, and that for $d_3$-EP was *m/z* 169 to *m/z* 151. The resolution of precursor-ions in Q1 and product-ions in Q2 was both set at 0.7 amu (FWHM). Argon was used as the collision gas and was set at 2.5 µTorr.



  

## Results and discussion

### Plausible cause of the diastereoisomeric conversion between EP and PE

In order to determine whether the phenomenon was matrix dependent or method dependent, the same set of methods applied to Sample 1 was repeated with another human urine sample (Sample 2) from a subject reportedly taken PE for medical purpose. Table 1 summarizes the results. Sample 2 was confirmed to contain only PE by both UHPLC-MS/MS (i.e. without derivatization) and TMS-GC-MS analyses. However, when Sample 2 was subjected to HFBA-GC-MS analysis, both EP and PE were detected (EP/PE = 7%). These findings strongly suggested that the diastereoisomeric conversion was specific to the HFBA-GC-MS method, and was likely to occur during HFBA derivatization. The same conversion during HFBA derivatization was also observed with just a reference standard of PE in the absence of the urine matrix.

Further studies were performed to investigate if other fluorinated acylating agents would give rise to a similar conversion. Two portions of a negative human urine sample were each spiked with either EP or PE (at 250 ng/ml each) and analyzed by GC-MS using TFAA, PFAA, or HFBA as the derivatizing agent. For comparison, these spiked samples were also analyzed using the aforementioned UHPLC-MS/MS and TMS-GC-MS methods. The isomeric purities of the EP and PE reference standards had previously been confirmed by UHPLC-MS/MS to be higher than 99%. As expected, while the analyses performed on the spiked samples using either UHPLC-MS/MS or TMS-GC-MS (Figures 1a and 1b) showed no diastereoisomeric conversion, considerable conversion was observed with HFBA-GC-MS (Figure 1c). Interestingly, the same conversion was also observed using PFPA and TFAA as derivatizing agents (Figures 1d and 1e), albeit to a lesser extent than that with HFBA. The extent of diastereoisomeric conversion appeared to be related to the degree of fluorination of the acylating agent, with HFBA giving the largest conversion (Table 1). This conversion was also not stereospecific as EP could be converted to PE and *vice versa*.

### Possibility of conversion at the GC inlet

To determine if the GC inlet would play a role in the diastereoisomeric conversion, the same HFBA-derivatized extract from a negative human urine spiked with 10 μg/ml of PE was injected a total of six times into three different GC-MS instruments on three separate days. The average EP/PE obtained from the repeated analyses was 42% and the RSD was only 3.9%. With such a small variation in EP/PE among different GC-MS instruments, the GC inlet is unlikely to have a significant influence on the conversion.

### Variation of the HFBA derivatization condition

In order to determine if the conditions of HFBA derivatization would have any effect on the diastereoisomeric conversion, experiments using different incubation time and temperature combinations were studied. Duplicate analyses were performed on extracts of a spiked human urine (at 10 μg/ml PE) derivatized with HFBA at: (1) 60 °C for 15 min; (2) 80 °C for 15 min; (3) 80 °C for 30 min; (4) 80 °C for 60 min; and (5) 80 °C for 120 min. To

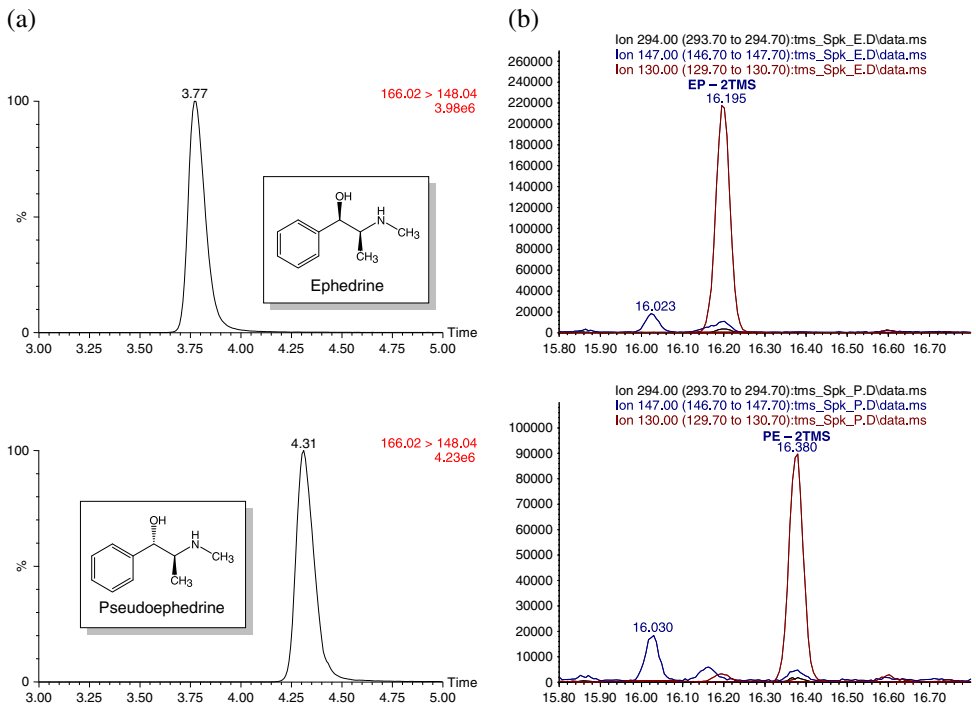


**Figure 1.** (a) Product-ion chromatograms of *m/z* 148 from the precursor-ion *m/z* 166 (protonated EP/PE) for urine samples spiked with EP (upper panel) and PE (lower panel) by UHPLC-MS/MS; (b) Extracted-ion chromatograms of *m/z* 294, *m/z* 147 and *m/z* 130 for urine samples spiked with EP (upper panel) and PE (lower panel) spiked urine samples by GC-MS after TMS derivatization; (c) Extracted-ion chromatograms of *m/z* 344, *m/z* 254 and *m/z* 210 for urine samples spiked with EP (upper panel) and PE (lower panel) spiked urine samples by GC-MS after HFBA derivatization; (d) Extracted-ion chromatograms of *m/z* 294, *m/z* 204 and *m/z* 160 for urine samples spiked with EP (upper panel) and PE (lower panel) spiked urine samples by GC-MS after PFPA derivatization; (e) Extracted-ion chromatograms of *m/z* 244, *m/z* 154 and *m/z* 110 for urine samples spiked with EP (upper panel) and PE (lower panel) spiked urine samples by GC-MS after TFAA derivatization.

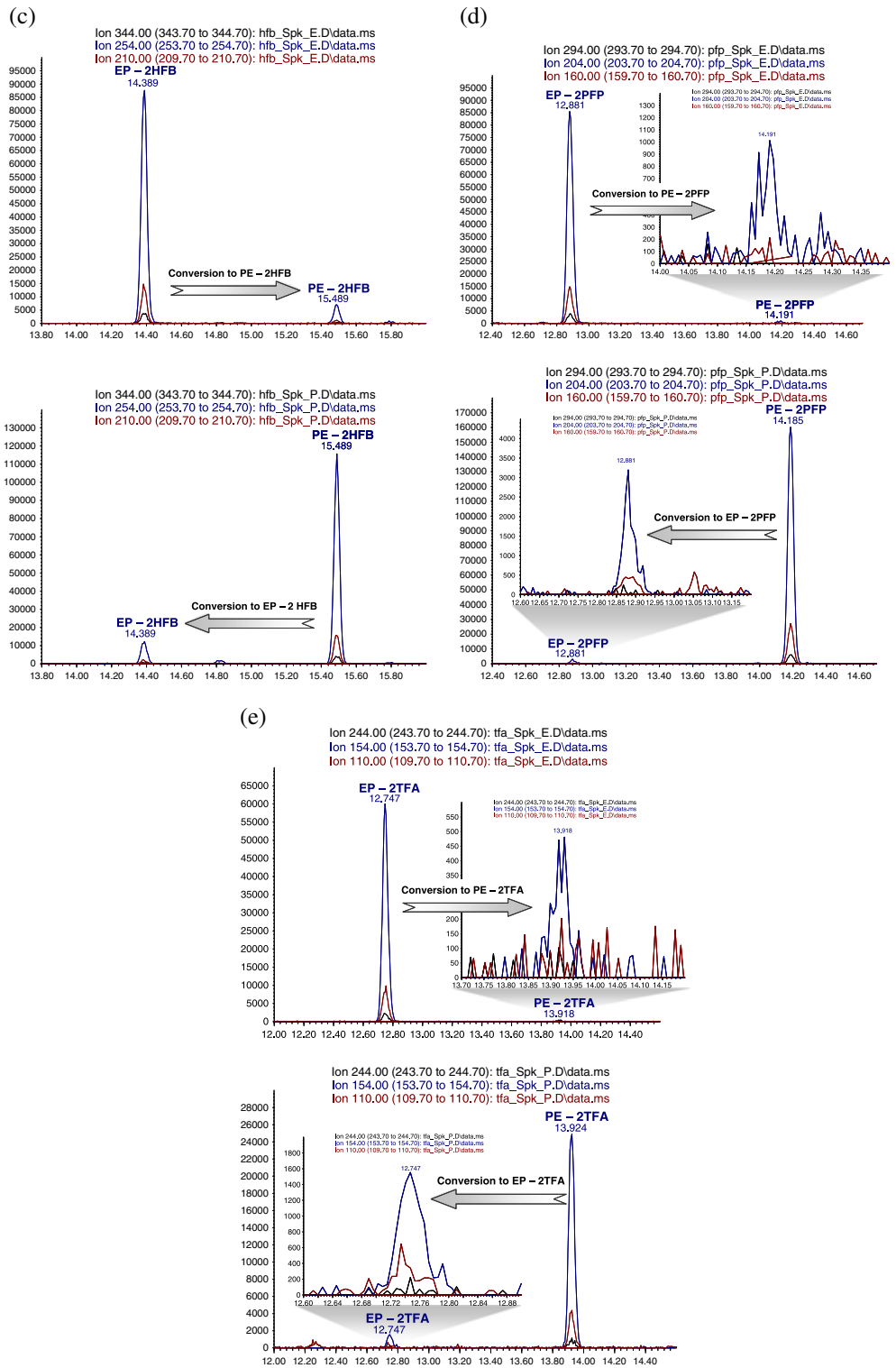


**Figure 1.** (Continued)

eliminate variations due to liquid-liquid extraction, the extracts were combined and aliquoted before derivatization. The results are summarized in Table 2. Due to the significant differences in EP/PE observed between some duplicate measurements, it was difficult to identify if there was a clear dependence of the extent of conversion on the incubation time or temperature. However, the average EP/PE obtained at 80 °C with different incubation times were about the same and slightly above that obtained at 60 °C for 15 min. Additional studies have been performed by repeating the experiment using (1) a different batch of HFBA derivatizing reagent, and (2) deionized water instead of urine spiked with pseudoephedrine at the same concentration. The results obtained were not very different (data not shown). In general, the results seemed to suggest that an incubation

**Table 2.** Variation of the HFBA derivatization condition on the extent of diastereoisomeric conversion

| Incubation condition | | EP/PE |
|---|---|---|
| (i) | 60°C for 15 min (A) | 5.3 % |
| | 60°C for 15 min (B) | 4.4 % |
| (ii) | 80°C for 15 min (A) | 7.9 % |
| | 80°C for 15 min (B) | 16.0 % |
| (iii) | 80°C for 30 min (A) | 6.0 % |
| | 80°C for 30 min (B) | 24.3 % |
| (iv) | 80°C for 60 min (A) | 6.6 % |
| | 80°C for 60 min (B) | 25.0 % |
| (v) | 80°C for 120 min (A) | 13.9 % |
| | 80°C for 120 min (B) | 10.5 % |

temperature of 80°C would give a higher extent of diastereoisomeric conversion than 60°C, and that an incubation time of 15 min or more would be sufficient to cause the conversion.

### Correlation between conversion yield and analyte concentration

The effect of analyte concentration on the extent of conversion was also studied. A PE post-administration human urine sample, in which the concentration of PE was estimated by UHPLC-MS/MS to be about 25 μg/ml, was analyzed in replicates of five after various dilutions (i.e. undiluted, 2.5-fold, and 5-fold dilution). The dilution was performed with the corresponding pre-administration urine blank. The internal standard, $d_3$-EP, was added to each aliquot at 100 ng/ml. The results are shown in Table 3. Although the mean EP/PE in the diluted samples (i.e. 2.5-fold and 5-fold dilution) were significantly lower than that without dilution, they were not directly related to their PE concentrations. Interestingly, the extent of conversion of the internal standard, $d_3$-EP to $d_3$-PE, also appeared to decrease with decreasing PE concentration (Table 3), although the concentration of the internal standard was the same for all samples studied. Based on these results, a direct correlation between the analyte concentration and the extent of diastereoisomeric conversion could not be established. However, the concentration of the available heptafluorobutyrate anion, might be a significant factor (please see the proposed mechanism below).

### Proposed mechanism for the diastereoisomeric conversion

To the best of our knowledge, the above diastereoisomeric conversion between EP and PE has not been reported previously. Since the extent of conversion appeared to increase with the degree of fluorination of the acylating agent, this phenomenon might be related to the propensity of the fluorinated-acyl moiety to act as a leaving group. This would explain why HFBA gave the highest conversion between EP and PE among the three acylating agents studied (i.e. HFBA, PFPA, and TFAA). Figure 2 shows a possible mechanism for the observed diastereoisomeric conversion. Using EP as an example, its bis-heptafluorobutyryl-derivative would undergo an intramolecular nucleophilic substitution at the benzylic carbon, with the oxygen of the amide moiety acting as the nucleophile. This is followed by ring opening of the resulting oxazolinium intermediate to give an achiral benzylic cation. Subsequent $S_N1$ substitution with the heptafluorobutyrate anion (which might be rate limiting) would lead to bis-heptafluorobutyryl-derivatives of both EP and PE.

## Conclusion

While HFBA had been reported as a useful derivatizing agent for the analysis of EP and PE by GC-MS because their corresponding derivatives could be completely separated by GC, this study showed that EP and PE could be interconverted during HFBA derivatization. Similar diastereoisomeric conversion was also observed with other fluorinated acylating agents (i.e. PFPA and TFAA). The extent of conversion appeared to increase with the degree of fluorination of the acylating agent, and a mechanism of the interconversion was proposed. These findings show that fluorinated acylating agents should not be used for the unequivocal identification or quantification of EP and PE as the results obtained can be erroneous.

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**Table 3.** Correlation between the analyte concentration and the extent of diastereoisomeric conversion

| *Urine Sample* | *PE (μg/mL)* | Mean ± SD of EP/PE (n = 5) | Mean ± SD of $d_3$-PE/$d_3$-EP (n = 5) |
|---|---|---|---|
| Sample A (no dilution) | ~ 25 | 15.5 ± 4.1 % | 20.2 ± 1.9 % |
| Sample A (2.5-fold diluted) | ~ 10 | 5.1 ± 0.5 % | 16.7 ± 3.7 % |
| Sample A (5-fold diluted) | ~ 5 | 7.7 ± 2.7 % | 14.9 ± 9.6 % |

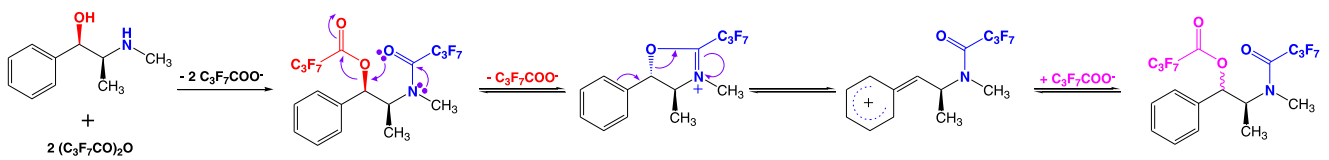


**Figure 2.** Proposed mechanism for the isomerization of bis-heptafluorobutyryl-EP.

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