Old School Derivatization in Modern LC-MS Analysis

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Derivatization

Modification of a chemical structure to improve its physiochemical properties

Since the early days used to:

- Detect spots on TLC plates
- Add chromophores to analytes prior to UV/VIS spectrometry
- Increase volatility in gas chromatography
- Increase detectability in HPLC-UV / FL

With the introduction of LC-MS, derivatization became "obsolete"

Why derivatizing when using LC-MS?

- Improve S/N ratio
  - Increase analyte detector response by introduction of a highly ionizable group
  - Different mass transition or switch of polarity with lower background noise
- Increase selectivity towards endogenous compounds
  - Chromatographic
  - MS
- Improve chromatographic retention
- Improve analyte stability
- Reduce autosampler carry over

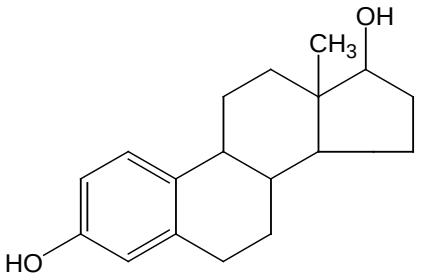
Procedure requirements

- Should fit into regular bioanalytical workflow
  - Not: reflux under argon during 24 hours at 195°C using a paladium catalyst.
- Readily available reagents
- No safety concerns
- Short reaction times (<1 hour)
- Stable derivative
- Efficient fragmentation
- Should lead to robust method
  - No highly critical steps

Tofofumi Santa, Biomed. Chromatochr., 210, 24, 915 – 918
Fengguo Xu, et al., Mass Spectrometry Reviews, 2011, 30, 1143-1172

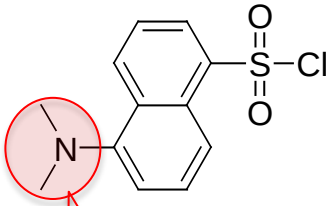
Example 1: phenolic hydroxyl

ethynylestradiol



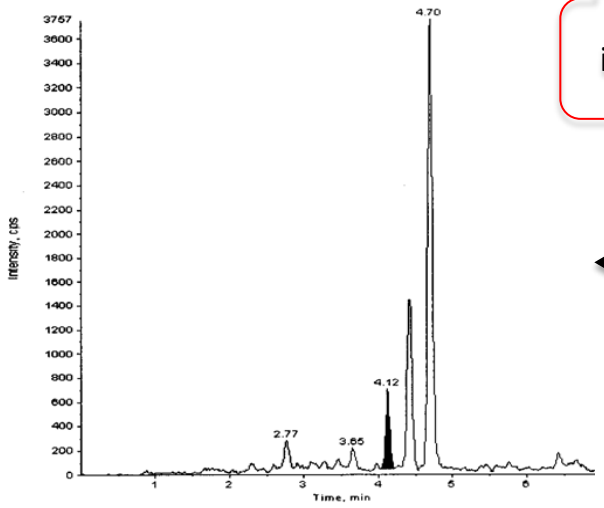
+

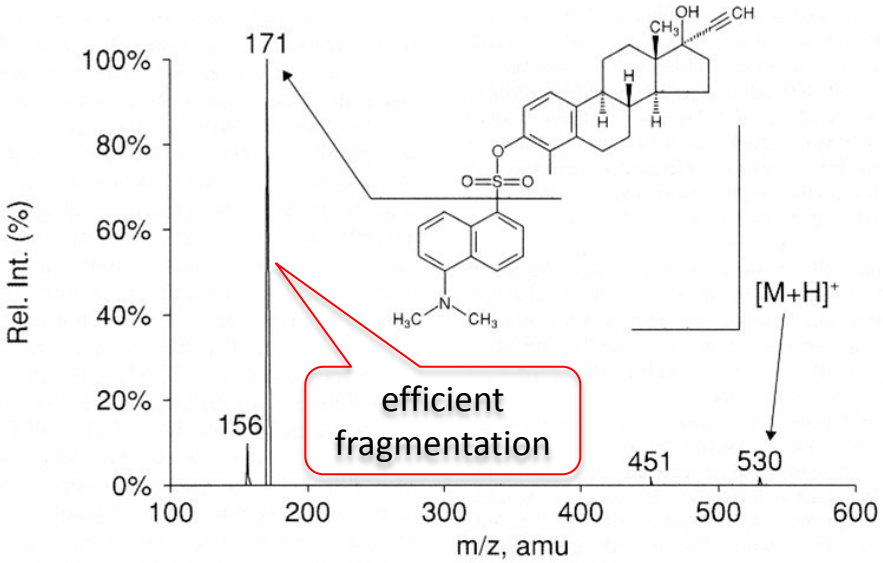
dansyl chloride



highly ionizable moiety

pH10.5
3 min 60°C

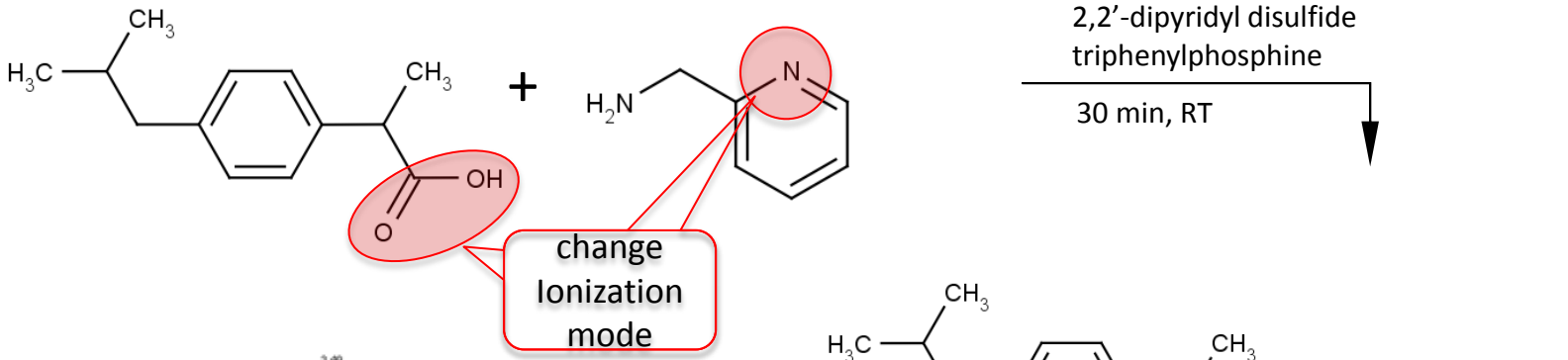


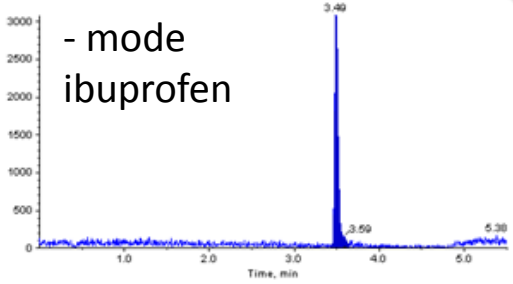


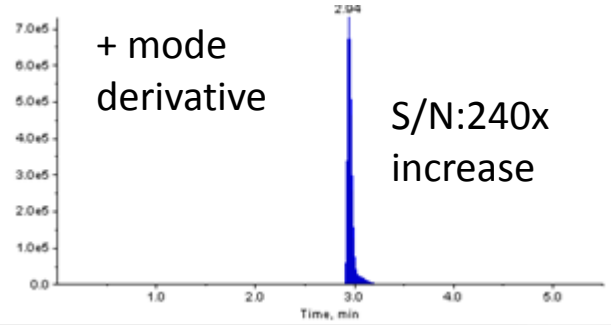
EE in plasma (2 pg/ml, API 4000, 100 µL plasma) ⟶ **80 fg on column**

Example 2: carboxylic acids

ibuprofen picolylamine



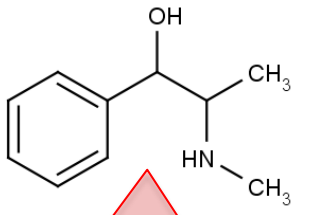




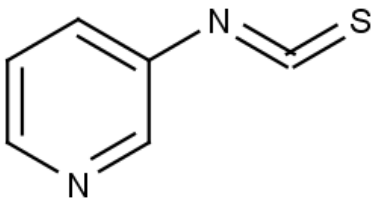
- From negative to positive ionization mode
- Improved ionization efficiency

Example 3: amines

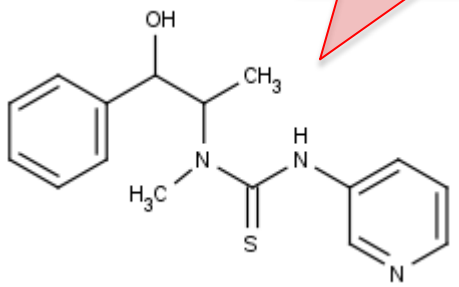
Ψ-ephedrine



3-Pyridyl isothiocyanate

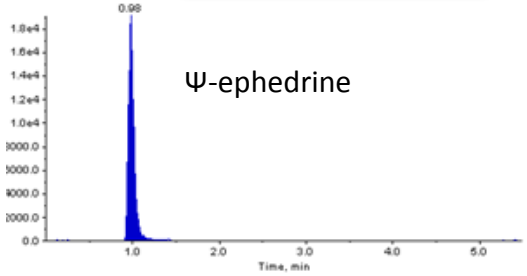


Pyridine
30 Min 60°C

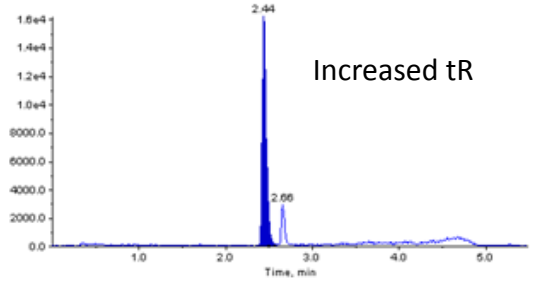


more retention

early eluting



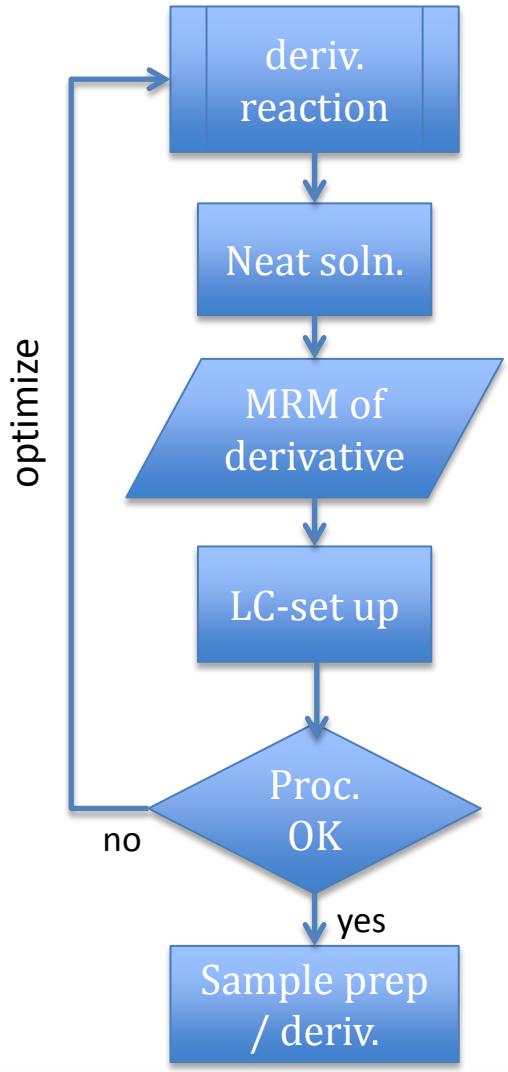
Ψ-ephedrine



Increased tR

- Better chromatographic retention
- Ionization efficiency unchanged

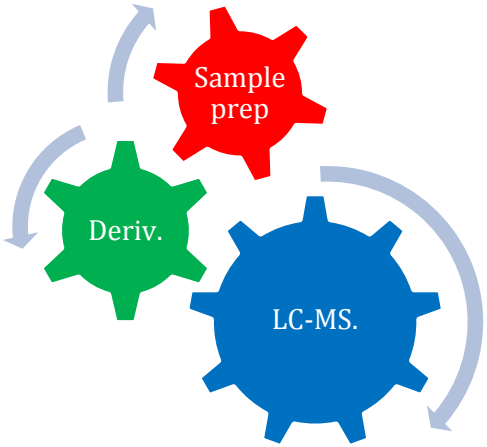
Method development perspective

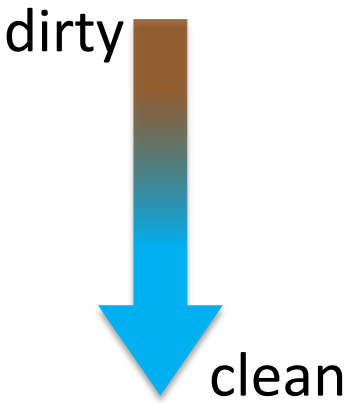


1. Select derivatization procedure
2. Prepare neat derivatized tune solution
3. Tune MS parameters of the derivative
4. Set up LC conditions with neat derivatized solution
5. Test / optimize derivatization in neat solutions
6. Develop extraction
7. Develop rest of the method

Method development perspective

- Optimize with respect to yield and proposed extraction procedure.
  - Temperature
  - Reaction time
  - pH / buffer type
  - Water content
  - Reagent concentration
    - cover full calibration range
    - presence of endogenous compounds
- Reaction medium:
  - Sample matrix
  - PP-supernatant
  - LLE-extraction solvent
  - SPE-elution solvent
  - Evaporated extract (residue)

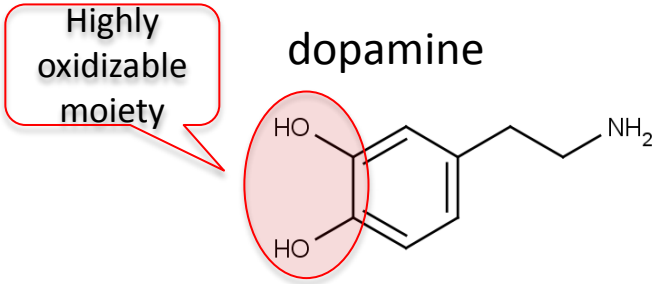




Application: dopamine in plasma

Issues (legacy from previous assay methods)

- Highly unstable
- Poor retention on RP-LC
- LLOQ: 50 pg/mL
- Selectivity issues, even with LC-MS

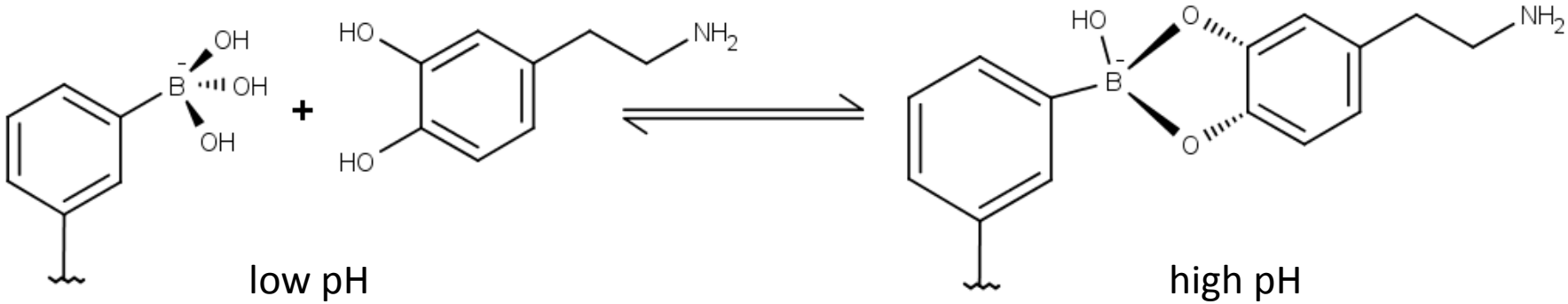


- Used to be:
  - SPE- SCX or LLE using phenyl boronic acid
  - RP-HPLC with electrochemical detection
  - Ion-pairing agent in mobile phase
- Goal:
  - Robust LC-MS/MS based assay using derivatization to address issues

Application: extraction

- Sample preparation by phenyl boronic acid-SPE
  - Highly selective reaction with cis-diol groups
  - Elution at low pH
  - Apply sample at pH 8.5, stabilized with glutathione and EDTA
  - Wash with 100% methanol
  - Elute with 1.0 mL of 0.1M HCl

temporary derivatization



low pH

high pH

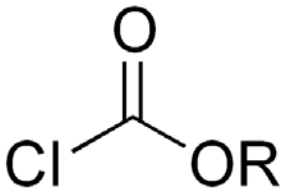
Application: derivatization

Requirements:

- Reaction in aqueous solution, preferably acidic
  - Catechol is stable at low pH
  - Avoid concentrating the SPE extract
- Reaction with both hydroxyl groups
  - Reduce oxidizability
  - Introduce hydrophobic groups to improve RP retention

Proposed reagent: Chloroformate ester

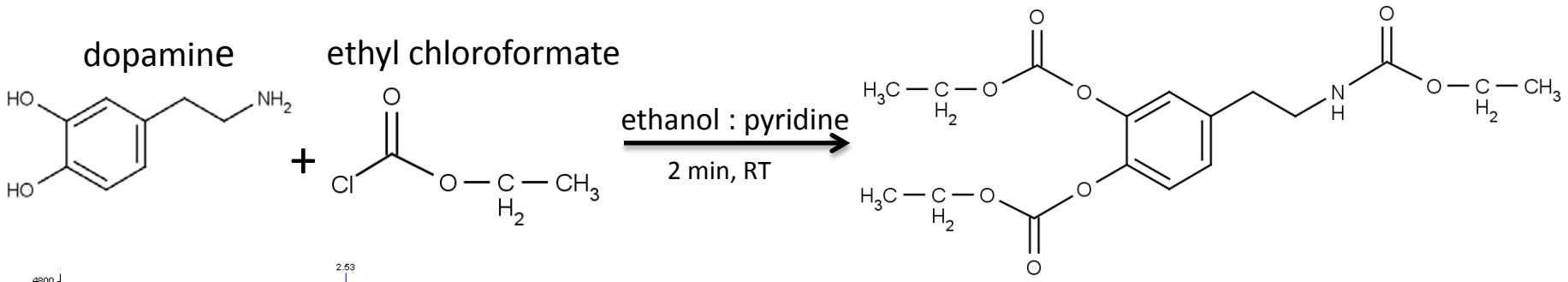
- Reacts with catechols and amines
- Reaction in aqueous medium
- Stable derivative
- Available with different side chains (R)

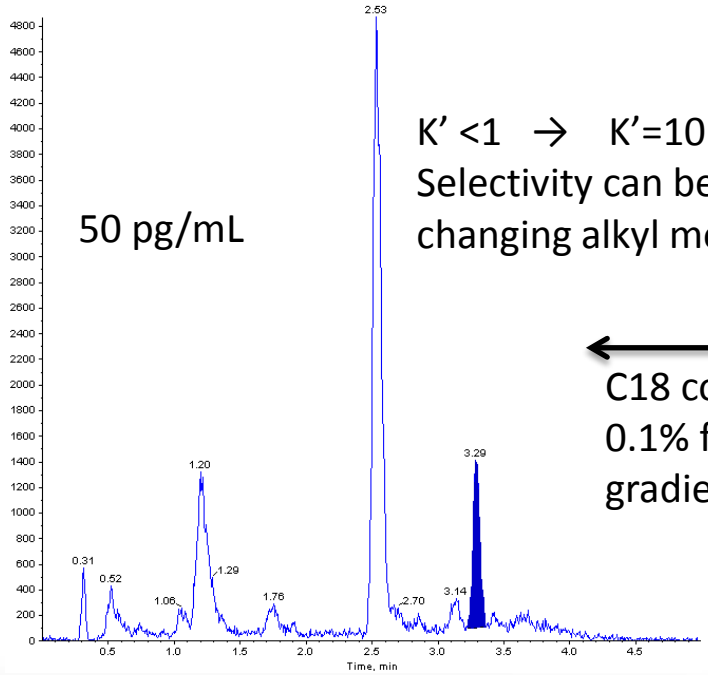


Application: derivatization

Derivatization direct in the SPE eluate:

dopamine + ethyl chloroformate → (ethanol : pyridine, 2 min, RT)





50 pg/mL

K' <1 → K'=10
Selectivity can be altered by changing alkyl moiety

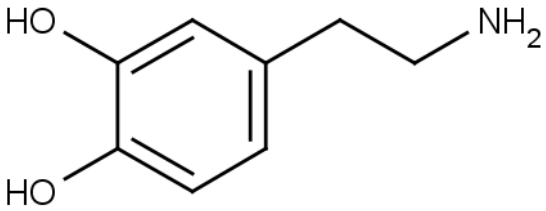
- LLE with T-BME
- Evaporation
- Recon. in small volume

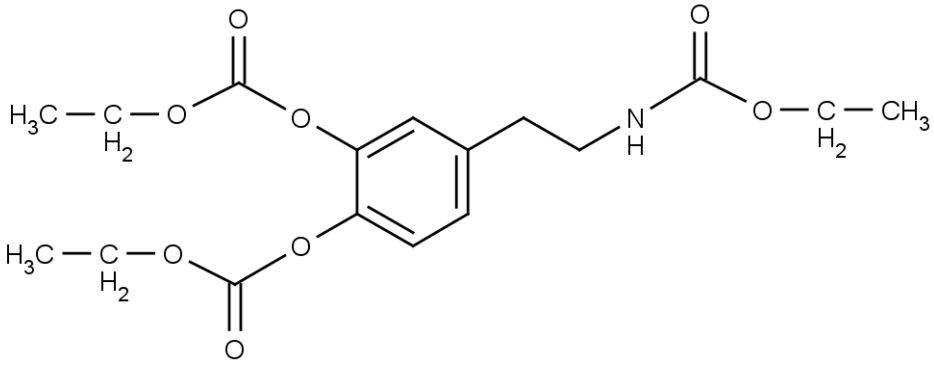
Extract

- Concentrated
- Clean
- Stable

C18 column
0.1% formic acid, methanol gradient

Application: results





BEFORE:

- Unstable (at high pH)
- Very hydrophilic
- Non-selective detection (MW 153)
- Insensitive (difficult fragmentation)

AFTER:

- Stable (entire pH range)
- More hydrophobic
- Selective detection (MW 392)
- Sensitive (easy fragmentation)

van de Merbel et al., Bioanalysis, 2011, 3, 1949-1961

Conclusion

Derivatization still is a very powerful tool to deal with a variety of challenges in modern LC-MS based bioanalytical method development

Acknowledgement

Method development team at PRA:

- Romy de Vries
- Harry Huisman
- Roderik Prins
- Peter Bults
- Kees Bronsema
- Martijn Hilhorst
- Nico van de Merbel