

Probing Electrospray Lipid Matrix Ionization Effects in the Analysis of Drugs in Plasma Samples

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Overview

- Phospholipids and lysophospholipids, which are present at very high levels in plasma, yield matrix effects in LC-MS/MS.
- "In-source multiple reaction monitoring" (IS-MRM) method developed for monitoring and probing lipid matrix ionization effects [1-2].
- Results were compared between methods which flushed lipids from the column to those that did not flush lipids from the column. In the latter case, lipids begin to "break-through" and coelute with analytes.
- Allowing lipids to "break-through" and coelute with analytes reduces sample cycle time, *i.e.* higher sample throughput

Introduction

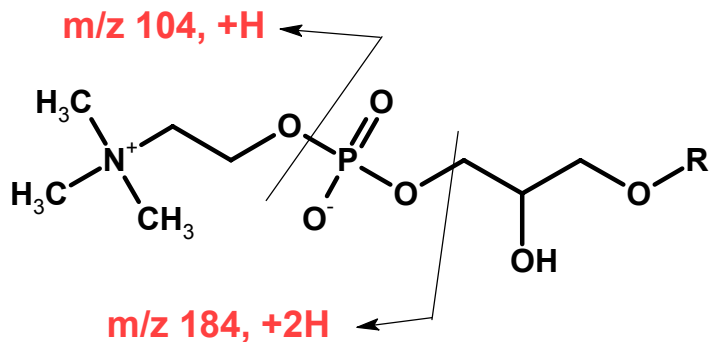
Endogenous lysophospholipids and phospholipids are present at very high levels in plasma samples. These lipids suppress or enhance the response of analytes in positive ion electrospray LC-MS/MS analyses [1-4]. Thus, it is essential that the effects of these lipids be evaluated during method development of quantitative analyses to support DMPK/ADME studies.

We have developed a simple method [1-2], which we refer to as "in-source multiple reaction monitoring" (IS-MRM) for detecting all phospholipids and lysophospholipids in one MRM channel. The approach was used to compare various chromatographic methods for the analysis of basic drugs in protein precipitated plasma. The lipids were either fully separated or coeluted with the analytes and quantitative results were compared.

General Experimental

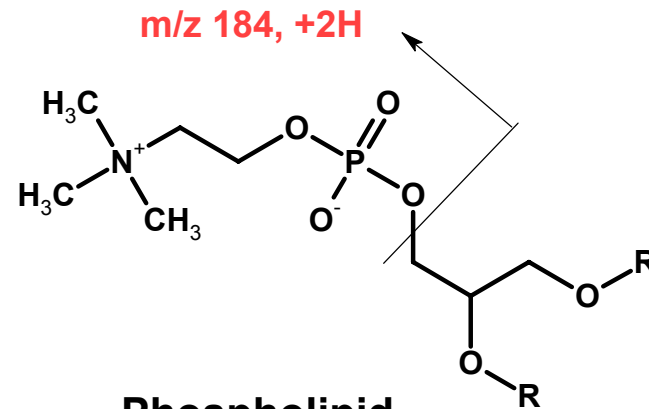
- IS-MRM: high energy *in-source* CID forms m/z 184 for all lipids of interest, Q1 and Q3 set at *same* mass to pass m/z 184 formed in source, collision cell at *low* energy to avoid further fragmentation, standard collision cell gas pressure
- IS-MRM on Quattro Micro with Acquity HPLC, 90 V cone voltage, 5 V collision cell energy; collision gas 3×10^{-3} mbar
- IS-MRM on 4000 Q Trap, DP 165 V, EP 10 V, CE 7 V, EP 5 V, collision gas pressure medium
- Protein precipitation of 100 μ l rat plasma with 250 μ l acetonitrile [6], samples centrifuged at 1800 x g in 96 well plates, inject from *above* protein pellet

IS-MRM Chromatogram for Lipids of Interest



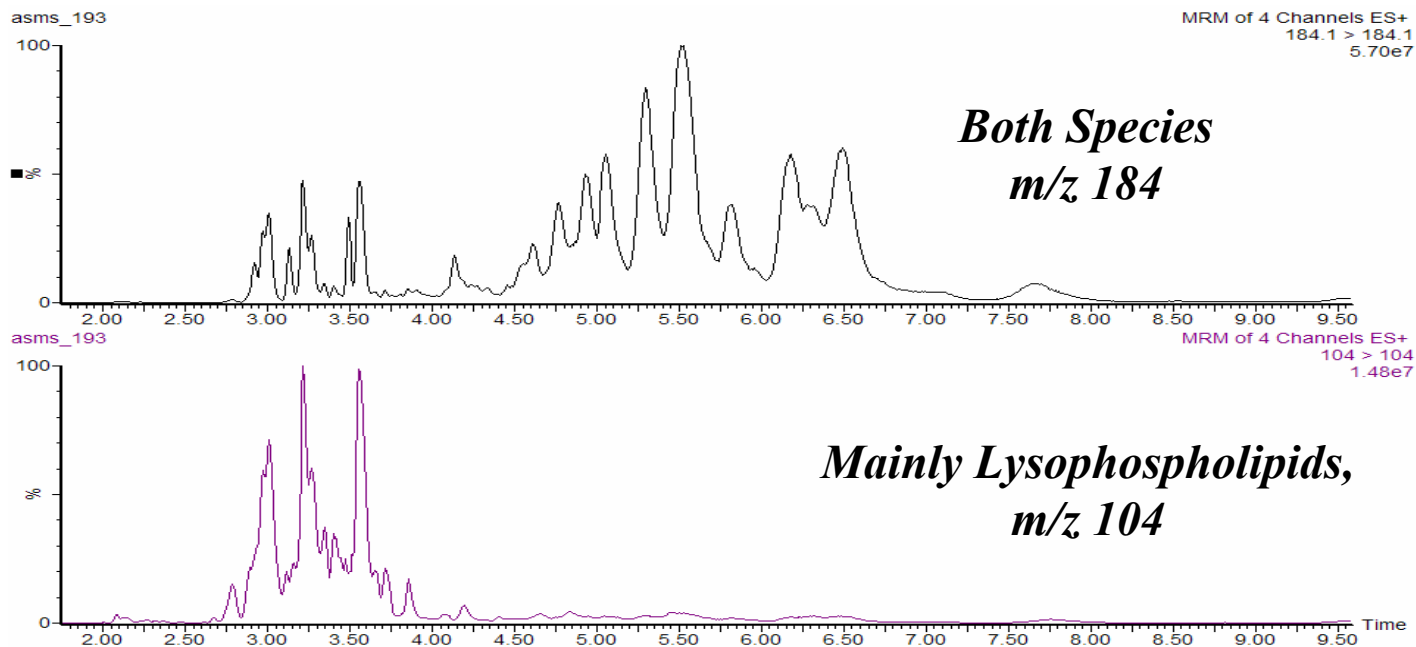
Lysophospholipid

R=Fatty acid ester, e.g. C18:0, C18:1, C16:0, etc.

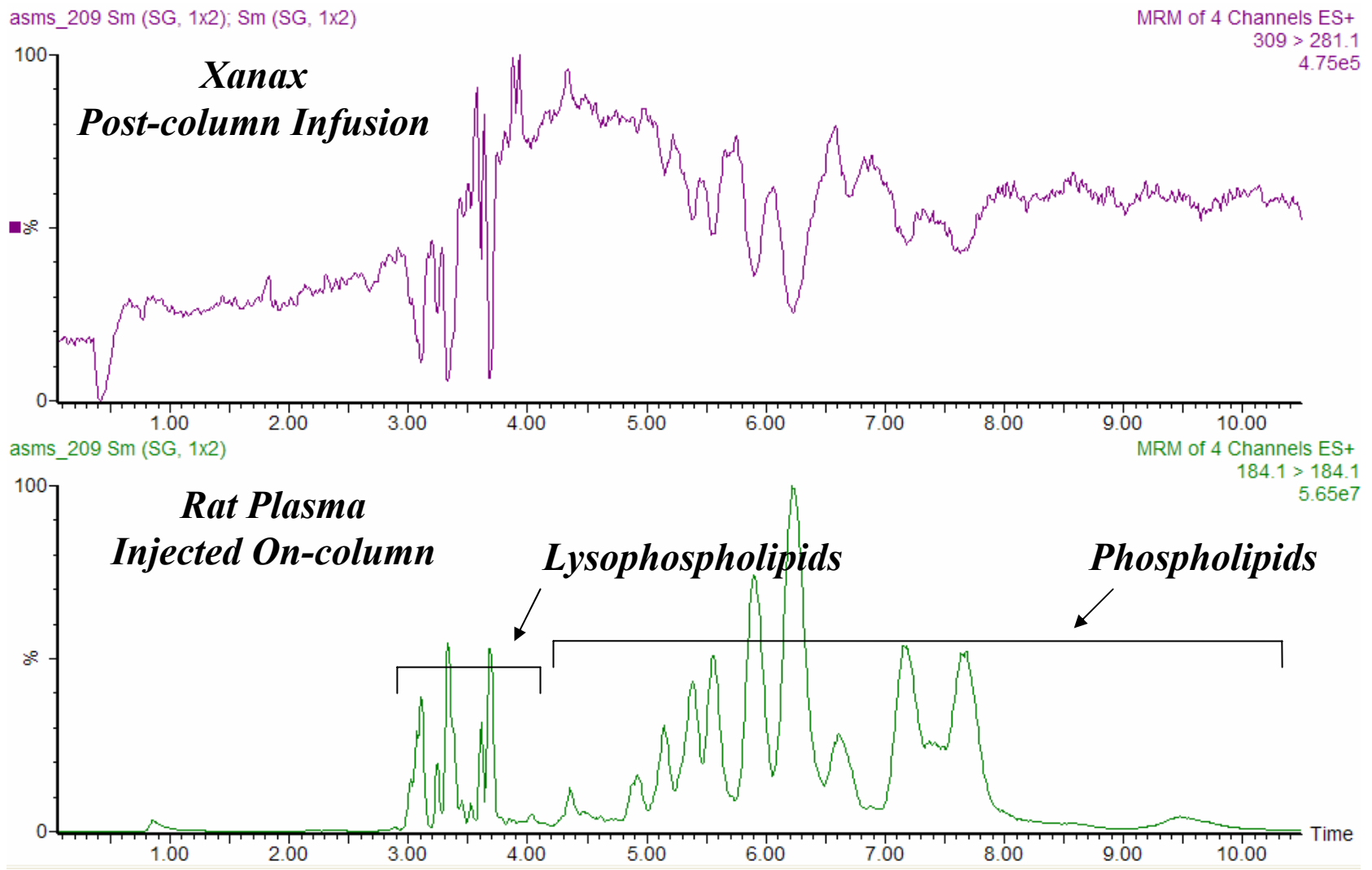


Phospholipid

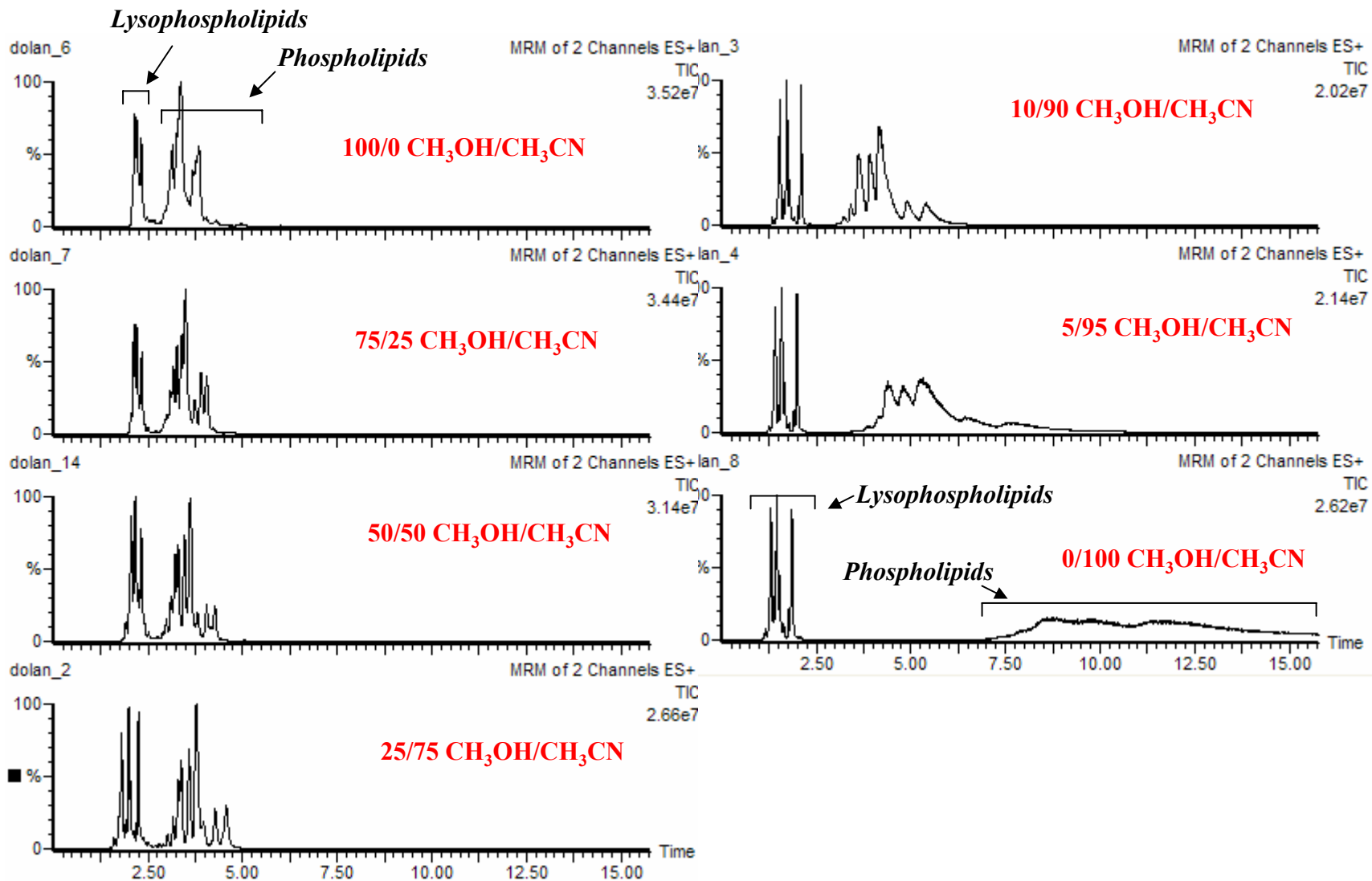
R=Fatty acid ester, e.g. C18:0, C18:1, C16:0, etc.



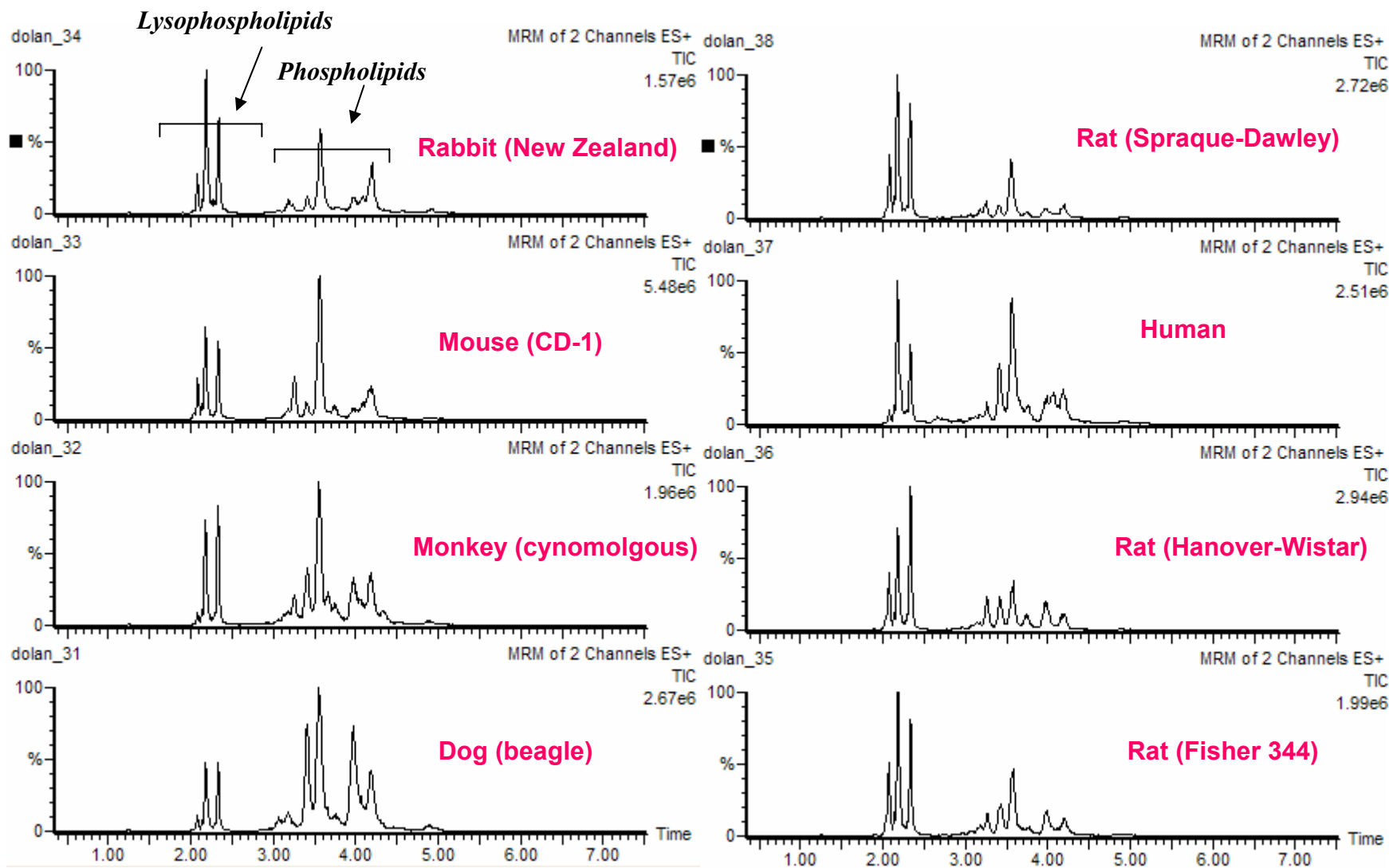
Matrix Suppression Shown by Post-Column Infusion of 180 ng/ml Solution of Xanax and Injection of Rat Plasma Sample



Solvent Selectivity *Very* Important in Eluting Lipids



Comparison of Different Species Using IS-MRM of m/z 184 and 104 Summed



Methods Evaluated

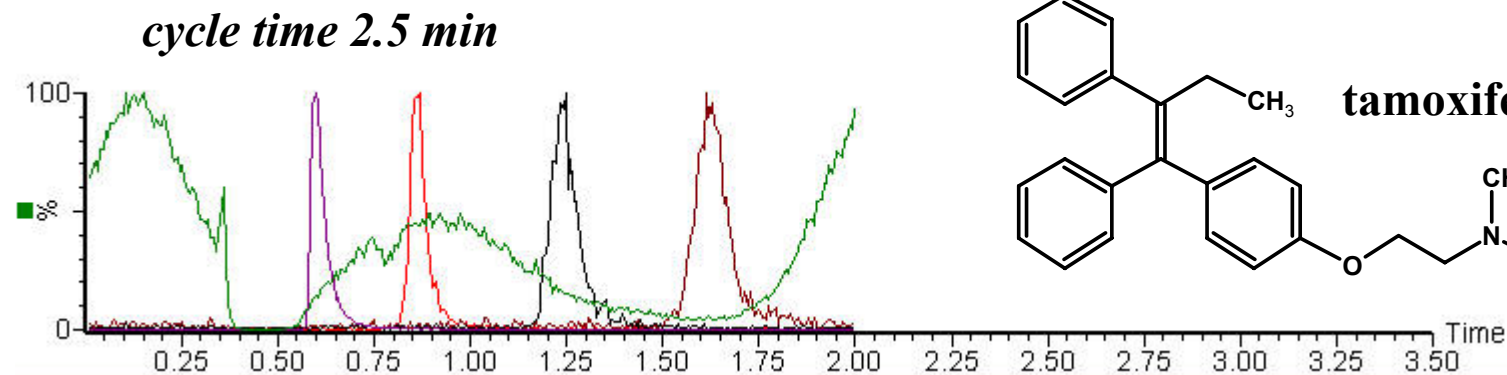
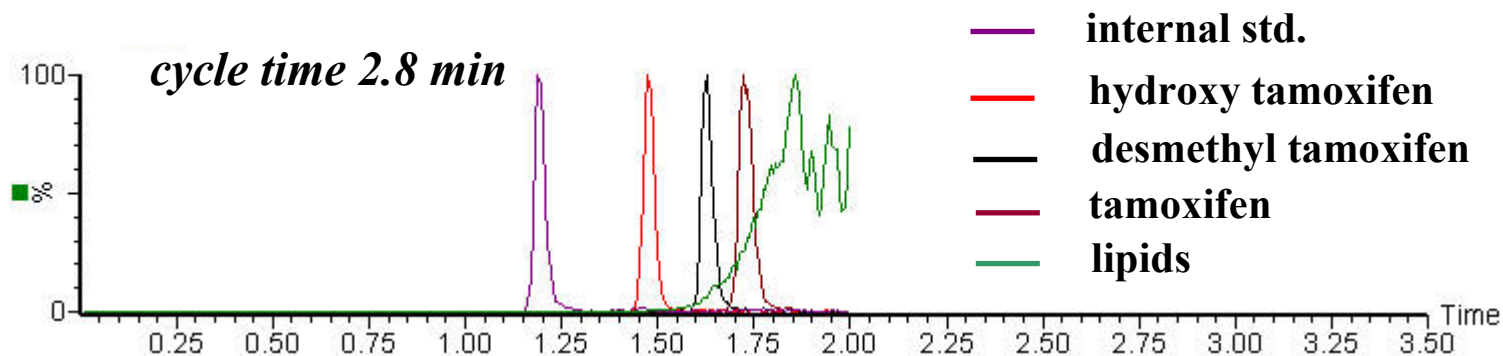
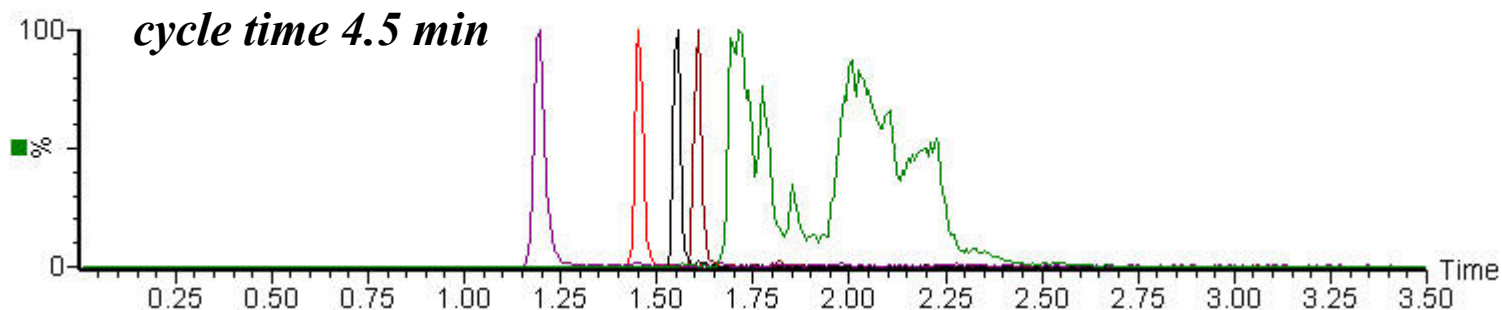
1. Standard gradient method for separating tamoxifen from lipids was used. The late-eluting lipids were diverted from MS and *totally* flushed from column during each separation.
2. "Steady state" gradient method for *separating* tamoxifen from lipids was used. Gradient cycled *immediately* to initial conditions after elution of tamoxifen. After 6-7 injections, late-eluting lipids began to "break-through" and their concentrations became essentially "steady-state." Quantitative analyses began *after* "steady-state" obtained.
3. "Steady-state" isocratic method for *separating* tamoxifen from lipids was used. The late-eluting lipids began to "break-through" after 6-7 injections. Quantative analyses began *after* "steady-state" obtained.
4. Standard gradient method for itraconazole using labeled internal std.
5. "Steady-state" isocratic method for itraconazole using labeled internal std.

Tamoxifen Method with Separation and Elution of Lipids

- 50 x 2 mm Varian Monochrom C18 HPLC column [5]
- Solvent A, H₂O 10 mmolar NH₄ formate adjusted pH ~4.5 + 3% MeOH
- Solvent B MeOH
- Method 1, full gradient shown below
- Method 2, gradient stopped after tamoxifen eluted, next injection after column equilibrated at 80% A
- Method 3, isocratic 25% solvent A, 75% solvent B, next injection after tamoxifen eluted

Time (min)	Flow (mL/min)	%A	%B
1. Initial	0.4	80	20
2. 0.20	0.4	80	20
3. 0.30	0.4	50	50
4. 1.30	0.4	0	100
5. 1.90	1.2	0	100
6. 3.51	1.2	0	100
7. 3.52	0.8	80	20
8. 4.00	0.4	80	20

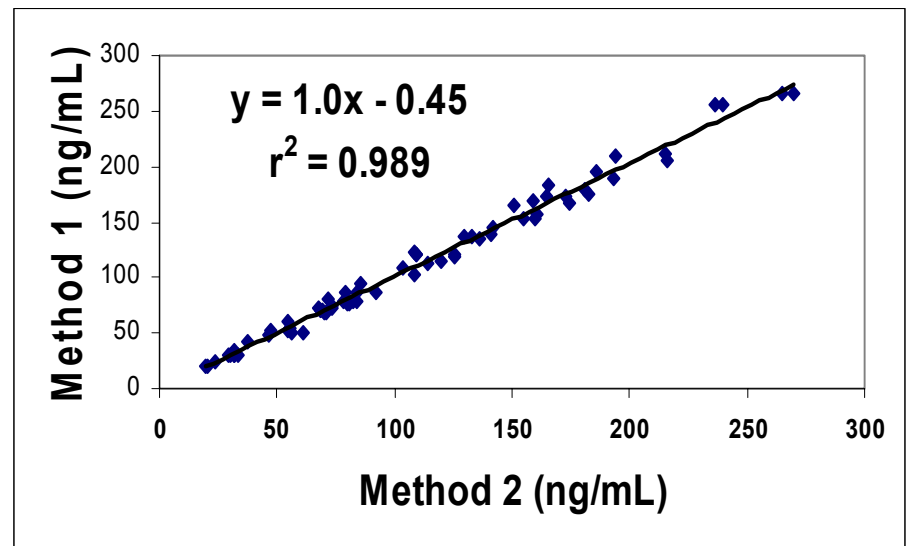
Comparison: Method 1 (Standard Gradient, *top*)
Method 2 (Steady-State Gradient, *middle*)
Method 3 (Steady-State Isocratic, *bottom*)



Comparison of Methods 1 and 2

- Methods gave similar results for tamoxifen and two metabolites
- FDA guidelines for calibrator and QC samples met
- "Steady-state" response for lipids varied 3% over 90 injections in PK study of 10 rats (7 time points/rat), 10 calibrators, and 10 QC's
- No effect on peak shapes or retention times for analytes
- Slopes of analytes calibration curves reproducible and increased, but no change in lower limit of quantitation
- Method 2 cycle time decreased ~40%

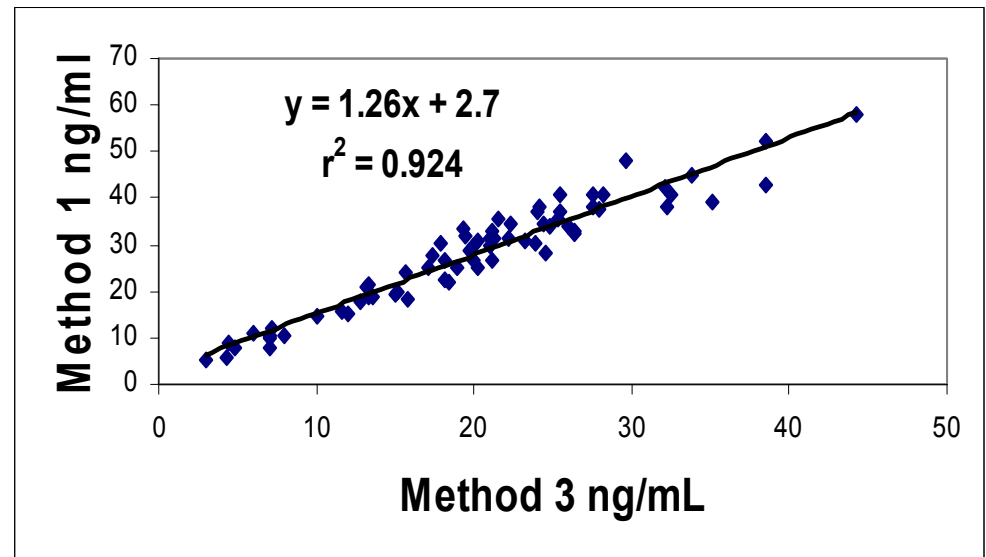
Comparison tamoxifen
results



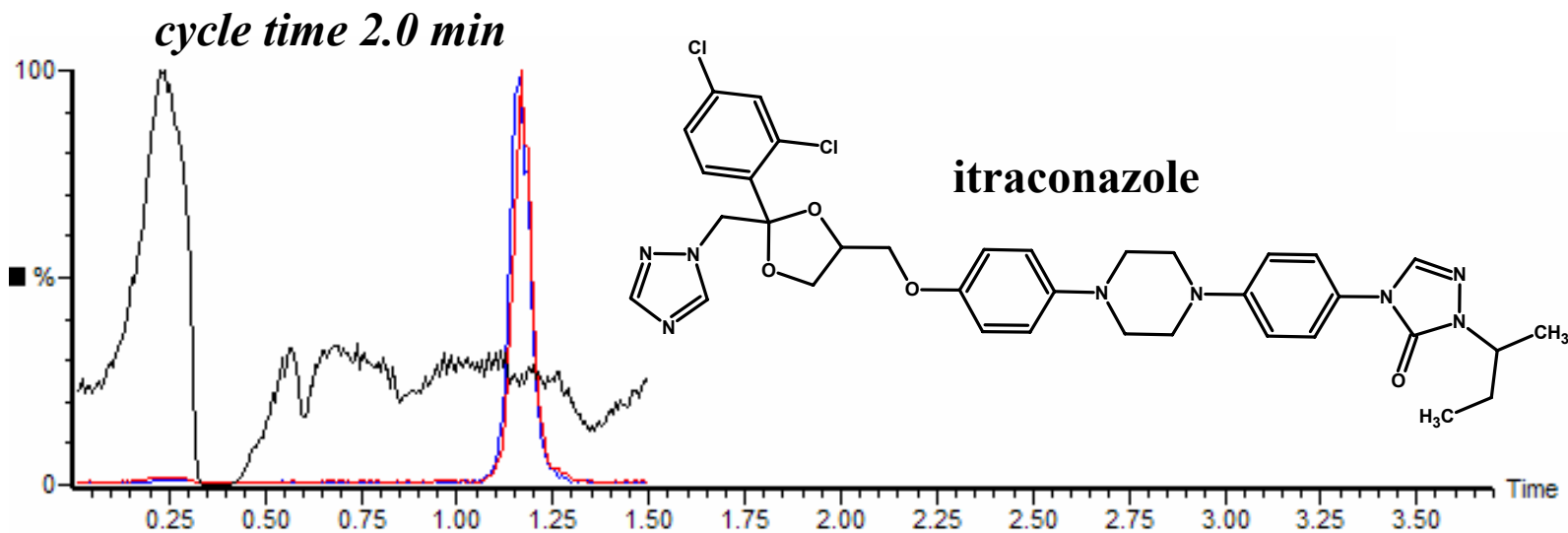
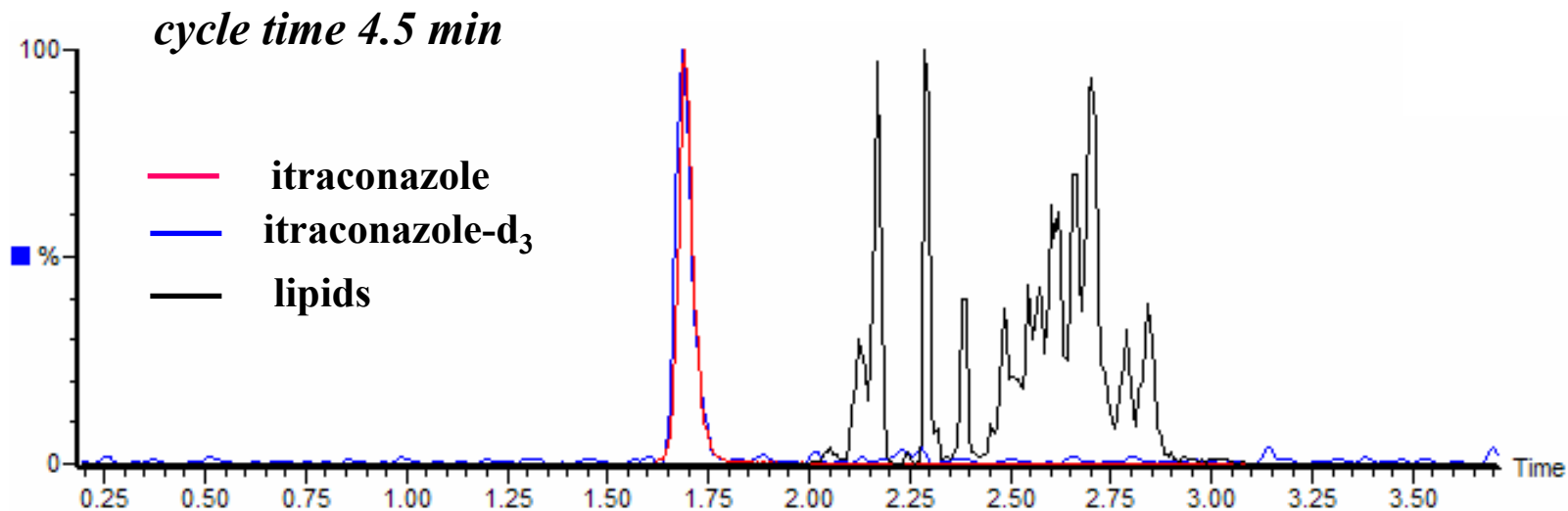
Comparison of Methods 1 and 3

- Methods did not give similar results for tamoxifen metabolites
- FDA guidelines for calibrator and QC samples not met
- "Steady-state" response for lipids varied 12% over 90 injections in PK study of 10 rats (7 time points/rat), 10 calibrators, and 10 QC's
- As expected, peak shapes broader for later-eluting analytes
- Slopes of analytes increased, lower limit of quantitation worse for later-eluting analytes
- Method 3 cycle time decreased ~45%

**Comparison
desmethy tamoxifen
results**



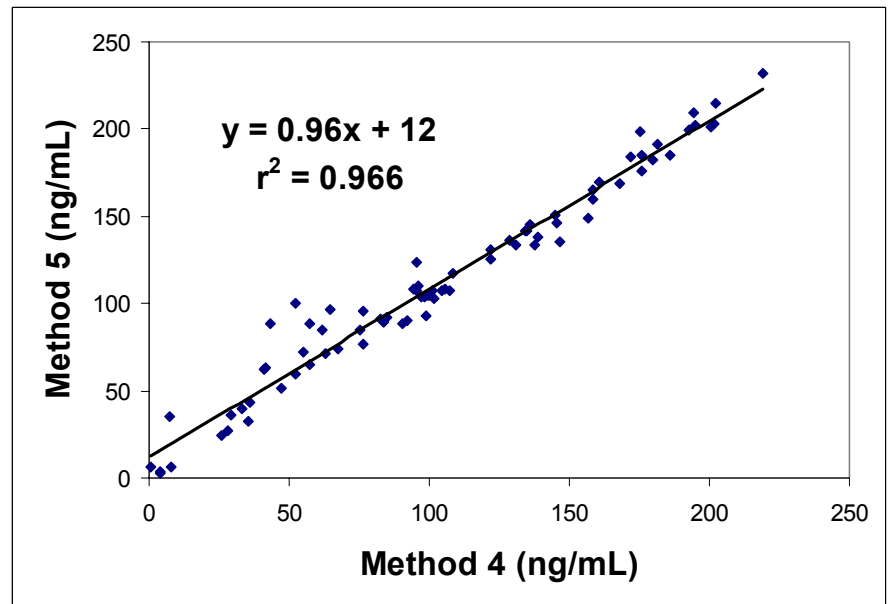
Comparison: Method 4 (Standard Gradient, *top*)
Method 5 (Steady-State Isocratic, *bottom*)



Comparison of Methods 4 and 5

- Methods gave similar results for itraconazole, but larger than desired bias in y intercept
- FDA guidelines for calibrator and QC samples met for 102 injections in PK study of 8 rats (10 time points/rat), 10 calibrators, and 12 QC's
- Responses for standard and internal standard changed as lipids began to "break-through," but response ratio remained constant
- Method 5 cycle time decreased ~55%

Comparison
itraconazole results



Discussion

Time delays between injections can lead to problems if the "steady-state" response noted for lipids changes. In the "steady-state" gradient (Method 2), no significant change was noted in the response for the lipids after an hour delay between injections. The initial gradient conditions keep the lipids focused on the column.

In contrast, the "steady-state" isocratic conditions (Method 3) will completely elute the lipids in ~40 minutes. This continual flushing of the lipids leads to an increased variability in the lipid concentration and the associated analyte response. Thus it will be more difficult to implement this latter approach.

The "steady-state" isocratic approach (Method 5) employing a labeled standard still has some biases, but does show some promise. The responses of the labeled and unlabeled standard do vary with the concentrations of the lipids; however, the response ratio is relatively constant.

Conclusions

Five different methods were evaluated using IS-MRM. The standard gradient methods separated the lipids from the analytes and the lipids were flushed from the column after each analysis. Several "steady-state" methods were evaluated in which the analytes are initially separated from the analytes, but not flushed from the column. After 5-6 injections, the elution profile and concentration of the lipids become somewhat "steady-state" and they coelute with the analytes.

The standard gradient method and the "steady-state" gradient method were found to yield essentially identical results for tamoxifen and two of its major metabolites. The overall sensitivity was not compromised, FDA guidelines were met, and the analysis cycle time reduced ~40%.

The "steady-state" isocratic methods would further reduce cycle times. However, biases were even noted with a labeled internal standard. However, the isocratic method does show promise and merits further development.

Currently we are employing the standard gradient approach for our analyses. The cycle times of <5 minutes meet our current sample load demands.

References

1. J. Little, ASMS 2005, San Antonio, TX.
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3. Patrick Bennett *et al*, phospholipids and lysophospholipids, www.tandemlabs.com/capabilities_publications.html
4. M. Ahnoff, H. Hagelin, ASMS 2004, Nashville, TN.
5. D. Humphries *et al*, J Chrom B, 810 (2004) 229-234.
6. P. Rudewicz *et al*, J. Chrom B, 799 (2004) 271-280.
7. Guidance for Industry, Bioanalytical Methods Validation, 2001, at www.fda.gov/cder/guidance/4252fnl.htm

Acknowledgements

We gratefully acknowledge John W. Dolan for valuable discussions and Stephen K. Haynes for sample preparation.

Available Information

Presentation and associated experimental detail available at:

"A Little Mass Spectrometry and Sailing,"

<http://users.chartertn.net/slittle/>

Website also contains information on:

- Matrix ionization effects in LC-MS/MS
- Identification of surfactants by LC-MS
- Using silylation and methylation reagents for GC-MS
- Accurate mass measurements by magnetic and TOF MS
- Versatile CI manifold for mixing and using gases
- GC-MS CI reagent gas selection
- NIST software for EI and MS/MS searches and data storage
- Polycarbonate and Polyester Analyses by GC-MS