ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
28 (2002) 279–285

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

# Comparison of SPE and fast LC to eliminate mass spectrometric matrix effects from microsomal incubation products

Joanna J. Zheng *, Eric D. Lynch, Steve E. Unger

*Department of Drug Metabolism and Pharmacokinetics, The DuPont Pharmaceuticals Company, Stine-Haskell Research Center, PO Box 30, Newark, DE 19714, USA*

Received 10 October 2000; received in revised form 14 March 2001; accepted 26 September 2001

**Abstract**

Twenty-seven highly diversified pharmaceutical compounds were used as a test set to evaluate matrix effects from microsomal media on LC/MS analyses. The individual effects of Tris buffer, NADPH and microsomes on ESI response were investigated. Direct flow injection MS/MS analysis, using no sample preparation or HPLC separation, gave an average of 2.2–5-fold matrix suppression in MS response from Tris buffer and NADPH. More polar analytes were affected the greatest. To reduce the loss in response, an automated solid phase extraction (SPE) procedure was developed. A much smaller average matrix effect was observed when samples were prepared using a Waters Oasis HLB 96-well SPE. As little as 1 ml of methanol (MeOH) was sufficient to elute most compounds with more than 80% recovery. Comparable results were obtained by directly injecting a protein-precipitated incubation onto a fast gradient LC separation prior to MS/MS detection. No advantage was seen by using both SPE and a fast LC separation prior to MS/MS analysis. 

*Keywords:* Matrix effects; Microsomes; In vitro; LC/MS; MS/MS

## 1. Introduction

As the combinatorial chemistry approach to drug discovery can synthesize hundreds or thousands of compounds per day, rapid and reliable screening methods are needed to select drug candidates with favorable human pharmacokinetic and safety credentials. In an effort of reduce costs and the use of animals, the application of in vitro drug metabolism data to understand the in vivo pharmacokinetic data has become an area of interest [1–4]. Several publications have demonstrated that, along with preclinical drug metabolism and pharmacokinetic data, human in vitro data can be used to predict human pharmacokinetic behavior [5,6]. Conventional HPLC cannot meet the challenge of analyzing large numbers of samples per day. To streamline and increase the throughput of in vitro metabolism assays us-

* Corresponding author. Tel.: +1-302-451-0024; fax: +1-302-366-5253.
*E-mail address:* joanna.j.zheng@dupontpharma.com (J.J. Zheng).

0731-7085/02/$ - see front matter 
PII: S0731-7085(01)00562-3

ing liver microsomes, high throughput LC/MS analysis have been developed and applied [7,8]. With the use of automation and rapid LC/MS analysis methods, screening large numbers of potential drug candidates can be accomplished in a relatively short time.

LC/MS analysis can resolve compounds having different molecular weights while MS/MS affords a second stage of spectrometric resolution, requiring specific fragments to be generated for analyte detection. Detection with minimum or no HPLC separation is possible. However, the biological matrix that co-elutes with the target analyte impacts MS response, particularly for electrospray ionization. These co-eluting species may be invisible to the MS detector. However, they can significantly affect the efficiency and reproducibility of the ionization process. Matrix effects are more prominent when minimal sample preparation and separation procedures are used, conditions preferred for fast analyses.

Published reports have shown the impact of urinary and plasma matrix effects on ionization efficiency for co-eluting components [9–11]. This paper reports an evaluation of matrix effects for microsomal incubation media. Since microsomal incubations are chemically complex, it is essential to understand the reason for ion suppression before providing an optimum analytical procedure. In this paper, 27 diversified pharmaceutical compounds were used in the evaluation of the matrix effects from microsomal media. Two procedures, an automated solid phase extraction procedure (SPE) and a fast gradient HPLC separation were developed and used prior to MS/MS analysis.

## 2. Experimental

### 2.1. Materials

Triprolidine, haloperidol, fenfluramine, carbamazepine, temazepam, prazepam, apomorphine and bromazepam were obtained from Sigma Chemical Co. (St. Louis, MO). The remaining compounds were obtained from various projects within the DuPont Pharmaceuticals Company and represent a wide diversity of structural types. Human microsomes were obtained from Xenotech (Kansas City, KS), while β-NADPH Tetra-Tris Salt and 50 mM Tris buffer (pH 7.3 at 37 °C) were purchased from Sigma Aldrich (St. Louis, MO). HPLC Grade Methanol (MeOH) and ACN were from Burdick and Jackson (Muskegon, MI).

### 2.2. Instrumentation

A Waters Oasis HLB 10 mg 96-well SPE plate (Waters, Milford, MA) was used for sample preparation. SPE was performed using a Zymark Robot, with Zymark custom 96-well centrifuge, Zymark 96-well evaporator (Zymark, Hopkinton, MA), and Tecan Genesis liquid handler (Research Triangle Park, NC). Flow injection analysis (FIA) using MS and MS/MS detection was performed with a Hewlett–Packard 1100 quaternary pump (Hewlett–Packard, Palo Alto, CA), Perkin–Elmer 200 autoinjector (Perkin–Elmer, Norwalk, CT) and Finnigan LCQ (Finnigan, San Jose, CA). High throughput LC/MS analysis was performed using Shimadzu LC pumps (Columbia, MD), Perkin–Elmer 200 autoinjector and Sciex API 3000 (Toronto, Canada).

### 2.3. Matrix effect evaluation procedures

A 100 μl solution containing 2 mM NADPH, 50 mM Tris buffer and 1 mg/ml human microsomes (same as the microsomal incubation) was placed in a 96-deep-well plate. An aliquot of 200 μl of ACN was added to one set of samples, as ACN was used to quench the incubation. Then, each of the 27 compounds was spiked into individual wells to make a final concentration of 1.7 μM. The precipitated proteins were removed from samples treated with ACN, by centrifuging the mixture at $156 \times g$ for 5 min. The supernatant of the mixture was directly injected into the LCQ for MS/MS analysis. Another set of the incubation cocktail was loaded onto preconditioned HLB SPE 96-well plates, washed with 1 ml of water, and eluted twice with 0.5 ml of MeOH. The combined MeOH eluate was evaporated under a $N_2$ stream at 37 °C. The residue was reconstituted in 300 μl of ACN:$H_2O$ (2:1), the same solvent

combination as that of the first set of samples. The test set was spiked into the reconstitution solution to achieve the same concentration as above. The same amount of compound was spiked into 300 μl of ACN:$H_2O$ (2:1) to serve as an unextracted solution for matrix comparison.

### 2.4. Automated SPE extraction

The 96-well SPE plate was placed on top of a Beckman square well plate on the deck of the Tecan liquid handler. The product from the microsomal extraction was placed into the designated position on the deck of the Tecan. In other cases, the microsomal incubation was carried out as the first part of the incubation and extraction process. The Tecan added 900 μl of MeOH to each well of the sample plate. Then, the plate was centrifuged at $100 \times g$ for 3 min prior to 900 μl of water being added to each well. The plate was centrifuged at $156 \times g$ for 3 min, completing the conditioning phase. The Tecan delivered the sample mixture to each well of the sample plate and the plate was centrifuged at $156 \times g$ for 3 min. The Tecan added 900 μl of water to each well and spun the plate at $156 \times g$ for 3 min. When needed, the washing procedure was repeated. The Tecan added 800 μl of MeOH to each well and the plate was spun at $156 \times g$ for 3 min. The Tecan added 200 μl of water to each well of the final collection plate to make the ratio of MeOH, water as 4:1 for

Table 1
Matrix effect of Tris buffer, and NADPH in the MS/MS analysis of 27 compounds

| Compound | Molecular weight | Protein precipitation | SPE |
|---|---|---|---|
| A | 335 | 0.04 | 0.59 |
| B | 352 | 0.25 | 0.76 |
| C | 612 | 0.22 | 0.89 |
| D | 411 | 0.19 | 0.68 |
| E | 481 | 0.46 | 0.98 |
| F | 384 | 0.07 | 0.41 |
| G | 476 | 0.31 | 1.01 |
| H | 460 | 0.25 | 0.98 |
| I | 259 | 0.17 | 0.58 |
| Indinavir | 613 | 0.38 | 1.31 |
| Nalfinavir | 567 | 0.29 | 0.90 |
| J | 667 | 0.22 | 0.84 |
| Vertex | 505 | 0.03 | 0.29 |
| K | 373 | 0.33 | 0.92 |
| L | 516 | 0.21 | 0.82 |
| M | 536 | 0.48 | 0.89 |
| N | 465 | 0.25 | 0.89 |
| O | 580 | 0.12 | 0.70 |
| P | 558 | 0.15 | 0.73 |
| Triprolidine | 278 | 0.30 | 0.73 |
| Haloperidol | 375 | 0.30 | 0.86 |
| Temazepam | 300 | 0.00 | 0.24 |
| Carbamazepine | 236 | 0.03 | 0.15 |
| Fenfluramine | 231 | 0.09 | 0.51 |
| Apomorphine | 267 | 0.25 | 1.08 |
| Bromazepam | 315 | 0.05 | 0.56 |
| Prazepam | 324 | 0.07 | 0.75 |
| Mean | | 0.20 | 0.74 |
| S.D. | | 0.13 | 0.27 |
| R.S.D. (%) | | 64 | 36 |

$n = 2$ injections, * ND, not determined.

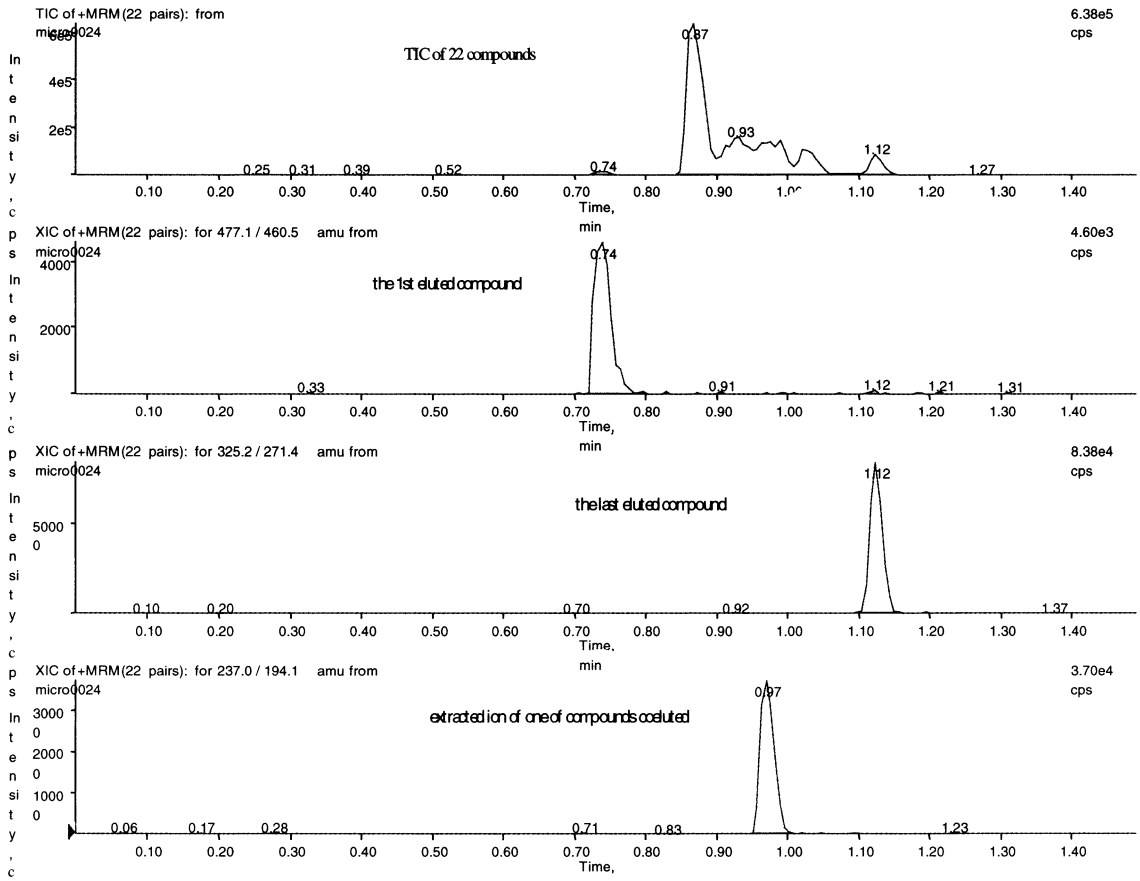


Fig. 1. Fast gradient chromatograms of test set TIC and representative extracted ions.

injection onto the LC/MS. During the extraction procedure, the Zymark arm delivered the plate combination to the centrifuge and back to the Tecan station.

### 2.5. FIA/MS/MS and LC/MS/MS analysis

Flow injection (no HPLC column) MS/MS analysis, The mobile phase was ACN, 0.1% FA/$H_2O$ (50:50) at a flow rate of 0.3 ml/min. No HPLC column was used for the matrix evaluation. For all compounds, sample peaks eluted around 0.2 min (dead time). The injection interval was 1.2 min (limited by autosampler). The samples were introduced into the electrospray source of the mass spectrometer. The temperature of the heated capillary of LCQ was set at 230 °C and nebulizer gas was set at 80.

High throughput LC/MS/MS analysis, fast gradient chromatography was performed on a Polaris C-18 (Metachem, 30 × 2 mm, 3 μ). The mobile phase consisted of 0.1% FA in $H_2O$ and ACN, starting at 10% ACN and increasing to 95% ACN over 0.8 min. The flow rate was 0.6 ml/min with a 1:1 split into the Sciex API 3000. The injection interval was 1.5 min (limited by re-equilibration). The temperature of Turbo Ionspray™ source was set at 400 °C. Auxiliary gas was set at 7 l/min, nebulizer gas at 9, curtain gas at 10 and CAD gas at 4.

## 3. Results and discussion

Microsomal incubations were generally quenched with ACN, and the supernatant was evaporated and reconstituted before injection into the MS. Any effect of microsomal proteins was mostly eliminated as proteins were precipitated with the addition of ACN. Buffer was suspected as the main cause for ionization suppression due to its high concentration and high ionic strength. We started by examining the matrix effect of Tris buffer and NADPH. Samples were individually injected into the mass spectrometer without a HPLC column to assess the worst possible matrix effect. The column labeled protein precipitation in Table 1 illustrates the results. The matrix effect was measured by dividing the peak area of the compounds in the matrix (post extraction) by that of the unextracted compounds. Ion suppression is indicated by results $< 1.0$, while enhancement is indicated by values $> 1.0$ MS/MS analysis of the test set in Tris buffer and NADPH using no sample preparation (protein precipitation) gave an average of 5-fold matrix suppression.

To reduce matrix effects, 3 approaches were considered, (1) use an alternative ionization method such as atmospheric pressure chemical ionization (APCI); (2) use a gradient HPLC separation; or (3) de-salt the analyte using SPE. While atmospheric pressure chemical ionization avoids competitive solution processes that impact ionization, it is generally less sensitive than ESI, can degrade thermally labile analytes or their metabolites and requires the molecule to have some degree of thermal stability and volatility. We wanted a procedure that was more generally applicable. To ensure the elution of a diverse set of analytes, gradient chromatographic procedures are required. We routinely use this approach and obtain best results with longer (5–6 min, first 1–2 min

Table 2
A comparison of matrix effect of the test set compounds in the presence of Tris buffer, microsomal proteins and NADPH

| Compound | Protein precipitation | | SPE | |
|---|---|---|---|---|
| | FIA number of LC column | LC with column | FIA number of LC column | LC with column |
| A | 0.30 | 0.83 | 0.81 | 0.84 |
| B | 0.43 | 0.80 | 0.64 | 0.75 |
| D | 0.52 | 0.81 | 1.09 | 1.22 |
| G | ND | 1.07 | 1.27 | 0.96 |
| H | 0.54 | 0.92 | 1.10 | 1.04 |
| Indinavir | ND | 1.05 | 1.16 | 1.19 |
| Nalfinavir | 0.60 | 0.83 | 0.92 | 0.98 |
| J | 0.42 | 0.82 | 1.01 | 1.16 |
| Vertex | 0.14 | 0.75 | 0.91 | 1.10 |
| K | 0.58 | 0.8 | 0.94 | 0.82 |
| L | 0.51 | 0.78 | 1.01 | 1.22 |
| M | 0.81 | 0.92 | 1.09 | 1.2 |
| N | 0.66 | 0.76 | 1.08 | 0.97 |
| O | 0.38 | 0.74 | 0.96 | 1.2 |
| P | 0.26 | 0.75 | 1.04 | 1.07 |
| Triprolidine | 0.51 | 0.72 | 0.73 | 0.75 |
| Haloperidol | 0.70 | 0.88 | 0.95 | 0.82 |
| Temazepam | 0.40 | 0.86 | 0.81 | 0.88 |
| Carbamazepine | 0.18 | 0.84 | 0.94 | 1.08 |
| Bromazepam | 0.44 | 0.92 | 0.99 | 1.13 |
| Mean | 0.46 | 0.83 | 0.93 | 0.98 |
| S.D. | 0.18 | 0.10 | 0.15 | 0.16 |
| R.S.D. (%) | 39 | 12 | 16 | 16 |

$n = 2$ injections, * ND, not determined.

Table 3
SPE extraction recovery of the test set from a matrix consisting of 2 mM NADPH, 50 mM Tris buffer and 1 mg/ml human microsomes

| Compound | Percent recovery |
|---|---|
| A | 100 |
| B | 81 |
| D | 83 |
| G | 96 |
| H | 91 |
| Indinavir | 92 |
| Nalfinavir | 88 |
| J | 81 |
| Vertex | 95 |
| K | 96 |
| L | 97 |
| M | 85 |
| N | 86 |
| O | 86 |
| P | 84 |
| Carbamazepine | 89 |
| Triprolidine | 93 |
| Temazepam | 84 |
| Bromazepam | 94 |
| Haloperidol | 91 |
| Mean | 90 |
| S.D. | 5.7 |

switched to waste) analytical run times. With the desire to achieve a higher throughput screen, the run time for each sample is ideally less that 2 min. With broad, generic gradients, run times are limited by re-equilibration of the HPLC system. Off-line extraction allowed us to minimize time spent on an expensive mass spectrometer.

We used both matrix effect and recovery determinations to guide the development of a generic SPE extraction method. After some initial screening of SPE sorbents, Oasis HLB was chosen. Both Tris buffer and NADPH are soluble in water and are washed from the SPE sorbent by strongly aqueous solutions. The improvement is illustrated by comparing the columns in Table 1 that are labeled protein precipitation and SPE (no microsomes). A much smaller average matrix effect (0.74) was observed when samples were de-salted using SPE. Using this procedure, only 4 of the 27 samples showed a matrix effect greater than 2-fold ( $< 0.5$). These four were generally more polar compounds. By using 96-well SPE prior to analysis, ESI response was significantly improved.

A return of a factor of four in ESI response afforded better detection sensitivity, allowing incubations to be undertaken at lower concentrations. In vitro determinations should be performed at concentrations below the $K_m$ of the substrate. We have observed suppression of ESI response as great as 10-fold, requiring either the use of more sensitive and expensive MS/MS instrumentation or better sample preparation and separation. Relative to pharmacokinetic studies, in vitro preparations are generally from the same source, so biological variability is minimized. However, as with analytical methods having low recovery, the impact of a strong matrix effect can also be to limit the precision and accuracy of the assay.

To understand the effectiveness of washing procedures, a set of samples was prepared and injected into the LCQ. ESI mass spectra from $m/z$ 100 to 900 were obtained to determine whether residual NADPH or Tris affected analyte response. Spectra following loading elution, as well as the first, second and third water wash steps were acquired. Responses from Tris and NADPH were selected from the spectra. The protonated molecules of Tris buffer at $m/z$ 122, reduced and oxidized forms of NADPH at $m/z$ 746 and 744, respectively, were observed. Following the second water wash, these three peaks were significantly reduced. It was determined that an additional water wash before elution afforded a cleaner sample. This final step optimized a simple, yet generally effective, preparation for microsomal incubations.

The matrix effect for 22 compounds was determined using either FIA (no column), or a fast gradient HPLC separation. A fast gradient elution with a short column was designed to give a $k' > 2$, allowing a similar on-line de-salting to the generic off-line SPE. Using a Sciex SPI 3000 and TurboIon™ source, a separation was established to elute all 22 compounds within 1.5 min (Fig. 1). Table 2 shows the results. Again, the matrix effect was largely reduced following the SPE procedure (0.93). By using a gradient LC column separation, the matrix effect was also reduced substantially (0.83). LC separation gave results that were simi-

lar to SPE; however, time for MS analysis is limited by the separation and chromatographic re-equilibration. No attempt was made to reduce injection cycle time for MS/MS applications. FIA or SPE MS/MS applications are limited by autosampler duty cycle. For some autosamplers, we have achieved injection cycles of 15 s. The lifetime of the LC column was not tested in this study. However, based upon our experience, it was suspected that the column backpressure would exceed a useful limit following a few hundred injections. The last column in Table 2 shows that the combination of SPE cleanup and LC separation showed little benefit over SPE alone (0.98 vs. 0.93). Given the lack of orthogonality of the two separation processes, this result was expected.

The recovery from the SPE extraction of the 22 compound test set was also obtained with LC/MS/MS analysis. The recoveries of the 22 compounds from the SPE range from 81 to 100% (Table 3). Since the test set covers compounds from many therapeutic areas at DuPont Pharmaceuticals, as well as commercially available compounds, the extraction protocol was considered generally applicable to future compounds with no or minimum modification.

## 4. Conclusions

This study provides useful information about the effects of microsomal matrix on ESI response for a test set of compounds. A general SPE procedure was developed that, along with the incubation, was fully automated. The Tecan combined with a Zymark robot can conduct sample incubation and SPE extraction on line, completing a 96-well plate within a few hours. While the extraction was carried out using an 8-probe liquid handler, 96-probe liquid handlers can accomplish the off-line extraction in a significantly shorter period of time. The time spent on the MS system is limited by the duty cycle of the autosampler, as the MS/MS analysis was accomplished without a HPLC column. Alternatively, injection of a protein precipitated sample with fast gradient HPLC gives comparable results, but is limited by the time needed to achieve the separation and gradient re-equilibration. No advantage is gained by using both SPE and HPLC separation prior to MS/MS detection.

## Acknowledgements

The authors wish to thank Thomas L. Lloyd, who was responsible for assembling the Zymark robotic system.

## References

[1] J.B. Houston, D.J. Carlile, Drug Metab. Dispos. 29 (1997) 891–922.
[2] T. Iwatsubo, N. Hirota, T. Ooie, H. Suzuki, N. Shimada, K. Chiba, T. Ishizaki, C.E. Green, C.A. Tyson, T. Sugiyama, Pharmacol. Ther. 73 (1997) 147–171.
[3] W. Kuhnz, H. Gieschen, Drug Metab. Dispos. 26 (1998) 1120–1127.
[4] R.S. Obach, Drug Metab. Dispos. 27 (1999) 1350–1359.
[5] R.S. Obach, J.G. Baxter, T.E. Liston, B.M. Silber, B.C. Jones, F. MacIntyre, D.J. Rance, P. Wastall, J. Pharmacol. Exp. Ther. 283 (1997) 46–58.
[6] T. Lave, S. Dupin, C. Schmitt, R.C. Chou, D. Jaeck, P. Coassolo, J. Pharm. Sci. 86 (1997) 584–590.
[7] R.B. van Breemen, D. Nikolic, J.L. Bolton, Drug Metab. Dispos. 26 (1998) 85–90.
[8] W.A. Korfmacher, C.A. Palmer, C. Nardo, K. Dunn-Meynell, D. Grotz, K. Cox, C.C. Lin, C. Elicone, C. Liu, E. Duchoslav, Rapid Commun. Mass Spectrom. 13 (1999) 901–907.
[9] I. Fu, E.L. Woolf, B.K. Matuszewski, J. Pharm. Biomed. Anal. 18 (1998) 347–357.
[10] D.L. Buhrman, P.I. Price, P.J. Rudewicz, J. Am. Soc. Mass Spectrom. 7 (1996) 1099–1105.
[11] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882–889.