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An Evaluation of a Four-Channel Multiplexed Electrospray Tandem Mass Spectrometry for Higher Throughput Quantitative Analysis

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A four-channel multiplexed electrospray inlet system (MUX) coupled to a triple quadrupole mass spectrometer was investigated as a higher throughput approach to quantitative analysis. Four discrete samples may be simultaneously analyzed by virtue of a rotating sampler with a concomitant 4-fold increase in analytical throughput. Although absolute sensitivity was reduced using the MUX interface compared with analysis using traditional single electrospray interface, reproducibility of response was shown to be comparable. Source robustness was established for the analysis of both aqueous drug standards and drugs in biological media, and linearity of response for a test compound, diazepam, was demonstrated over 2 orders of magnitude. Analyte-dependent response differences were exhibited between the four channels of the interface, and this led to the overall conclusion that samples to be compared quantitatively must be analyzed through the same sprayer. In addition, each channel must be independently calibrated to afford true quantification. Should a deuterated internal standard be employed, however, quantitative comparisons can be made across channels. An HPLC pumping system providing individual back-pressure regulation to each channel was shown to provide adequate chromatography even in the event of a channel blockage. Furthermore, following multiple injections of biological samples onto the MUX interface, an eluent flow diversion was integrated into the first part of each analytical run. This served to prevent source fouling, and thus, no detrimental effects to response reproducibility or sensitivity were observed.

The application of recent technological advances to the drug discovery process such as combinatorial chemistry[1−4] and high-throughput screening[5,6] has resulted in the ability to produce large numbers of diverse compounds for routine screening against numerous targets. This has led to a change in focus from lead discovery to lead optimization. As a consequence, there is increased demand for the early evaluation of drug metabolism and pharmacokinetic (DMPK) properties in candidate molecules with the aim of reducing the potential for failure in drug development due to poor DMPK properties.[7]

The routine application of HPLC−mass spectrometry as a bioanalytical tool in the drug discovery environment, due to the technique's high sensitivity and selectivity for the analyte of interest over the background matrix, is well documented.[8,9] In recent years, the enhanced selectivity of tandem mass spectrometry has been exploited for the simultaneous analysis of compound mixtures. Employing such instrumentation has enabled the support of multiple compound "cassette" dosing for the rapid evaluation of pharmacokinetic properties[10−14] or postdose sample pooling following single-compound administration.[15−17] However, the analytical and experimental caveats[18−21] associated with these approaches limit their effectiveness in providing definitive PK information.

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Alternative approaches to higher throughput evaluation of drug metabolism center around increasing the pace at which samples obtained from such studies are prepared for analysis. This has been achieved through the introduction of new techniques such as automation of sample preparation by the application of microtiter plate technology and the adoption of automated liquid handling.[22−27] Additionally, there is increasing evidence for direct injection of plasma samples for LC−MS/MS analysis either by on-line sequential SPE[28−31] or by the use of so-called "turbulent flow chromatography".[32,33] One consequence of this is that the ability to process samples for analysis is no longer rate limiting such that the attention is now switched back to providing methodologies to expedite the mass spectrometric analysis. The objective is to provide timely and relevant drug metabolism information that will impact directly on drug design; hence, an ideal solution would therefore be to couple instrumentation providing enhanced analytical capacity that takes advantage of automated sample preparation. Such instrumentation, however, should be without the drawbacks associated with the simultaneous analysis used for cassette dosing.

A recently emerging approach toward increased mass spectrometric throughput in support of drug discovery is the introduction of multiple inlet electrospray interfaces.[34−38] Here, an electrospray source capable of sampling multiple liquid streams from individual chromatographic systems allows the parallel analyses of multiple samples. This is achieved by virtue of a rapidly rotating aperture, such that, at any one time the spray from a single channel only is introduced to the mass spectrometer.

The work described herein is an evaluation of a four-spray interface coupled to a tandem mass spectrometer. The instrument is operating in multiple reaction monitoring (MRM) mode for quantitative analysis. Key questions in the use of this technology are how it compares in terms of sensitivity, reproducibility, and robustness relative to traditional single electrospray analyses. The potential role for such technology in the bioanalytical arena should also be established.

EXPERIMENTAL SECTION

Materials. The analytes diazepam, diazepam-d_5, nitrazepam, and clozapine were supplied by Sigma-Aldrich (Poole, U.K.). Ammonium formate was obtained from Fisher (Loughborough, U.K.). Acetonitrile (HPLC grade) and dimethyl sulfoxide (DMSO; AnaLar grade) was supplied by BDH (Poole, U.K.). Formic acid (SuperPurity grade) was supplied by Romil Ltd. (Cambridge, U.K.). For the in vitro incubations, rat liver microsomes were prepared in-house from livers of male Sprague-Dawley rats (B&K, Hull, U.K.) by standard methodology[39] and evaluated for protein content[40] and cytochrome P-450 content.[41] Incubation medium was Dulbecco's phosphate-buffered saline (DPBS) sourced from Life Technologies (Paisley, U.K.) and the cofactor NADPH tetrasodium salt was obtained from Calbiochem (Nottingham, U.K.).

Liquid Chromatography−Mass Spectrometry. All mass spectrometry was performed using a Micromass Quattro Ultima (Micromass, Manchester, U.K.) fitted either with a Z-spray ESI (single channel) source or with a four-channel MUX interface. All experiments were conducted in positive ion MRM mode. The instrument was set to unit mass resolution on Q1 and Q3.

Generic MS/MS conditions were used for all analyses: source temperature 120 °C, desolvation gas temperature 350 °C, cone gas nitrogen 30 L/h, desolvation gas nitrogen ~800 L/h. Capillary voltage was set to 3.2 kV for both MUX and single ESI analyses. MRM transitions for each of the analytes were determined by infusion (20 μL/min, 5 ng/μL); however, it is possible to expedite this process by using the QuanOptimize software package (Micromass), which affords automated MS/MS optimization by flow injection analysis via the MUX interface. MRM transitions were monitored as follows: diazepam 285.2 → 193.1, cone voltage 60 V, collision energy 30 eV; diazepam-d_5 290.3 → 198.4, cone voltage 60 V, collision energy 30 eV; nitrazepam 282.4 → 236.2, cone voltage 50 V, collision energy 20 eV; clozapine 327.3 → 270.2, cone voltage 35 V, collision energy 20 eV. Dwell times for each transition were 100−150 ms, and for the MUX analyses, the instrument was set to 50-ms interdwell delay and 50-ms interspray step time. MassLynx version 3.5 was used for data acquisition and signal processing. Microsoft Excel 97 was used for data manipulation and calibration curve construction.

The HPLC system comprised a Jasco PU-1500 binary HPLC pumping system with a vacuum degasser connected to four Jasco PU-1585 individually back-pressure-regulated channel pumps (Jasco, UK Ltd., Essex, U.K.). The autosampler was a CTC HTS-PAL equipped with a four-way Harney valve upgrade (Presearch, Herts, U.K.). The system is composed of a single syringe which sequentially preloads the four injection valves. These valves are then switched simultaneously to afford parallel analysis of four discrete samples. A programmable four-way Valco divert valve was incorporated to divert the first minute of each run to waste to avoid source fouling (Thames Restek UK Ltd., Berks, U.K.).

HPLC columns were Ace (50 × 2.1 mm i.d., $d_p = 3$ μm) from Advanced Chromatography Technologies (Aberdeen, U.K.). The

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mobile phase consisted of 25 mM ammonium formate buffer, adjusted to pH 3.0 with formic acid, and the organic modifier was acetonitrile. Chromatography was performed using a generic gradient (t = 0 min, MeCN 20%; t = 0.3 min, MeCN 80%; t = 2.0 min, MeCN 80%; t = 2.1min, MeCN 20%. Total run time including all injector valve washes, 4 min). For the MUX analyses, the flow rate at the binary pump was set to 2.7 mL/min and the individual channel pumps were set at 0.5 mL/min each. The eluent from each of these channels was split postcolumn to 100 μL/min by virtue of adjustable T-piece splitters. The remaining flow was diverted to waste. In the case of the single ESI work, the same HPLC gradient was used but the flow rate at the binary pump was set at 0.9 and 0.5 mL/min at a single channel pump. This was introduced into the source without splitting. All injection volumes were 20 μL for MUX analyses and 10 μL for single ESI.

Sample Preparation. *Pure Standards.* Analyte stock solutions were prepared in 1/1 v/v 25 mM NH_4COOH adjusted to pH 3.0 with HCOOH/acetonitrile. Diazepam calibration curve standards were prepared at seven concentrations: 0.025, 0.05, 0.1, 0.25, 0.5, 1, and 2.5 μM with either 5 μM nitrazepam or 1 μM diazepam-d_5 as internal standard.

In Vitro Incubations. Diazepam was incubated at 1 μM in rat liver microsomes (0.5 mg/mL total protein) in a shaking water bath (15 min, 37 °C). Total incubation volume was 2 mL in the presence of excess cofactor (NADPH, 2 mM). The incubation medium was DPBS. The reaction was terminated by addition of an equal volume of acetonitrile (2 mL). Incubations were performed in triplicate and control incubations were performed in the absence of cofactor. NADPH was then added back to the samples following termination of the incubations. Samples were centrifuged (2000 rpm, 15 min) and the resulting supernatant injected onto the LC−MS/MS system described previously.

MUX Sensitivity and Reproducibility Experiments. The absolute sensitivity and reproducibility of MUX analysis was compared with single ESI analysis. Here, a solution of 0.5 μM diazepam was analyzed via both interfaces; injections (10 μL, n = 12) were performed using the ESI source and injections (20 μL, n = 12) per channel using the MUX source. In a similar experiment and following the same protocol, a solution containing 0.5 μM diazepam with 0.1 μM clozapine was injected. The aim of this experiment was to determine analyte-dependent differences between channel responses.

MUX for Quantitative Analysis. Diazepam calibration standards prepared at seven concentrations 0.025, 0.05, 0.1, 0.25, 0.5, 1, and 2.5 μM with 5 μM nitrazepam as internal standard were injected through each channel of the MUX interface. Each calibration set was injected five times to establish response reproducibility. This experiment was repeated with a second set of calibration standards which contained 1 μM diazepam-d_5 as internal standard.

Application of MUX Analysis to Quantitative Bioanalysis. Samples obtained following incubation of diazepam under oxidative conditions with rat liver microsomes were analyzed using the MUX interface. Triplicate incubations were performed in the presence and absence of the cofactor NADPH. Each incubation and control set were injected three times down each channel. Average peak areas were calculated and the microsomal turnover calculated using the following equation:

$$\text{microsomal turnover (\%)} = 100 - ((\text{peak area diazepam}_{(+\text{NADPH})} / \text{peak area diazepam}_{(-\text{NADPH})}) \times 100)$$

To investigate instrument robustness, a diazepam stock solution (0.5 μM) was repeatedly injected (n = 24 injections/channel) both prior to and immediately after injection of a series of microsomal incubates (n = 24 injections/channel).

Table 1. Comparison of Absolute Sensitivity and Reproducibility of MUX Analysis with Single ESI Analysis for Repeat Injections of a Single Solution of Diazepam (0.5 μM)[a]

analysis	channel	mean peak area ± SD	CV (%)	relative response MUX/ESI (%)
MUX[a]	1	36 104 ± 1940	5.4	2.5
MUX[a]	2	34 070 ± 1151	3.4	2.4
MUX[a]	3	43 054 ± 1825	4.2	3.0
MUX[a]	4	41 030 ± 820	2.0	2.9
ESI[b]		7 100 773 ± 37022	5.2	

[a] Data are average peak area data ± SD for 12 20-μL sequential injections per channel for MUX analysis. [b] Data are average peak area data ± SD for 12 10-μL sequential injections for single ESI analysis.

RESULTS AND DISCUSSION

MUX Sensitivity and Reproducibility Experiments. A comparison of single ESI and MUX interfaces suggests that for single MRM analysis (diazepam, 0.5 μM) the reproducibility of data obtained via each channel of the MUX interface is comparable between channels. A coefficient of variation (CV) of between 2% and 5% per channel was observed (Table 1), which is similar to that generally achievable with a conventional single electrospray source (CV = 5%, Table 1).

In the case where a mixture of two analytes was analyzed by both MUX and single ESI interfaces (diazepam and clozapine at concentrations of 0.5 and 0.1 μM, respectively), good intrachannel reproducibility was again demonstrated for the MUX analyses (Table 2). This CV also compared favorably with single ESI as described above. However, large analyte-dependent differences between channel responses were observed. The resultant effect is that the interchannel variability for the peak area ratios (peak area$_{(\text{diazepam})}$/peak area$_{(\text{clozapine})}$) has a coefficient of variation in excess of 30%. This cross-channel variability is clearly not acceptable for quantitative analysis and suggests that for true quantification the separate channels must be calibrated individually. This is a general observation of the work described herein and a point that is discussed in greater detail below.

The overall sensitivity of the MUX analyses compared poorly to single ESI, with channel dependent responses of just 3%−8% of that achieved for single ESI. This reduction in sensitivity is somewhat higher than previously documented,[37] where losses in the order of 3-fold were demonstrated between ESI and MUX analyses. Some of this reduction in sensitivity may be attributed to the fact that that postcolumn splitting is essential. This is because the maximum solvent flow rate that may be delivered to

Table 2. Demonstration of Compound-Dependent Differences in MUX Channel Responses for Two Analytes, Diazepam and Clozapine[a]

	peak area diazepam			peak area clozapine			peak area ratio	
channel	mean ± SD	CV (%)	rel resp (%)	mean ± SD	CV (%)	rel resp (%)	mean ± sd	CV (%)
1	20 404 ± 1531	7.5	4.3	43 328 ± 3069	7.1	7.2	43 328 ± 3069	7.1
2	25 649 ± 1768	6.9	5.4	30 121 ± 2275	7.6	5.0	30 121 ± 2275	5.6
3	28 086 ± 1702	6.1	6.0	47 019 ± 1415	3.0	7.8	47 019 ± 1415	7.6
4	18 988 ± 1073	5.7	4.0	19 368 ± 1252	6.5	3.2	19 368 ± 1252	5.2
interchannel variability	23 282 ± 4297	18.5		34 959 ± 12675	36.3		34 959 ± 12675	32.1

[a] Data are average peak area ± SD for 12 20-μL sequential injections/MUX channel of a single solution of diazepam (0.5 μM) and clozapine (0.1 μM). Peak area ratio is calculated as peak area$_{(diazepam)}$ /peak area$_{(clozapine)}$ and rel resp is the relative peak area response per MUX channel with respect to each analyte as compared with that obtained for 12 sequential ESI analyses of the same solution (diazepam, 471 686 ± 22 935; clozapine, 599 988 ± 41 498).

Table 3. Linearity of MUX Analysis for a Series of Diazepam Standard Solutions[a]

	diazepam/nitrazepam (IS)			diazepam/diazepam-d_5 (IS)		
channel	slope	intercept	r^2	slope	intercept	r^2
1	1.7212	−0.0354	0.992	1.3854	0.0021	0.996
2	1.3003	−0.0076	0.997	1.3519	0.0368	0.997
3	1.1151	−0.0160	0.995	1.4132	0.0233	0.998
4	1.3611	0.0235	0.998	1.3599	0.0335	0.997

	diazepam/nitrazepam (IS)		diazepam/diazepam-d_5 (IS)	
interchannel variability (slope)	mean slope ± SD	CV (%)	mean slope ± SD	CV (%)
	1.3744 ± 0.2538	18.5	1.3776 ± 0.0278	2.0

[a] Calibration curve statistics for (A) diazepam (0.025−2.5 μM) with 5 μM nitrazepam as internal standard (IS) and (B) diazepam (0.025−2.5 μM) with 1 μM diazepam-d_5 as internal standard. Large interchannel variations in the slopes for the diazepam with nitrazepam as internal standard curves are diminished with the deuterated diazepam internal standard.

Table 4. Reproducibility of MUX Analyses for a Series of Diazepam Standard Solutions[a]

	diazepam/nitrazepam (IS)		diazepam/diazepam-d_5 (IS)	
channel	mean slope ± SD	CV (%)	mean slope ± SD	CV (%)
1	1.8365 ± 0.1334	7.3	1.4048 ± 0.0864	6.2
2	1.3876 ± 0.1406	10.1	1.4490 ± 0.0735	5.1
3	1.2369 ± 0.1236	10.0	1.4609 ± 0.0517	3.5
4	1.5798 ± 0.1512	9.6	1.4296 ± 0.0654	4.6
interchannel variability (channels 1−4)	1.5102 ± 0.2589	17.1	1.4361 ± 0.0245	1.7

[a] Average calibration curve slopes ± SD obtained for five sequentially analyzed sets of standards of either (A) diazepam (0.025−2.5 μM) with 5 μM nitrazepam as internal standard (IS) and (B) diazepam (0.025−2.5 μM) with 1 μM diazepam-d_5 as internal standard.

the MUX interface is around 0.4−0.5 mL/min. In this case, 20% of the eluent flow (0.1 mL/min per channel) was supplied to the MUX interface and 80% was diverted to waste, whereas for the single ESI experiments the total flow (0.5 mL/min) was delivered to the source. However, experiments where the same flow split was introduced to single ESI demonstrated a negligible drop in signal response (data not shown). In addition, further evidence to support this was obtained from experiments where three of the four MUX channels were disconnected and the eluent flow through the remaining channel was introduced without splitting. This gave no improvement in channel response (data not shown) and implies that the reduction in sensitivity observed is not due to the total amount of sample introduced to the source. Instead, it is more likely to be a function of the source design and perhaps the fact that fewer data points are collected across a chromatographic peak.

MUX for Quantitative Analysis. Pure standard stock solutions of diazepam at seven concentrations over 2 orders of magnitude (0.025, 0.05, 0.1, 0.25, 0.5, 1, and 2.5 μM) were injected through each MUX channel. Nitrazepam (5 μM) was included as internal standard was also present in each standard. The peak area ratios achieved, and the correlation coefficient $r^2 \geq 0.992$ (Table 3) clearly demonstrates response linearity across this concentration range for each of the MUX channels. However, the slopes of the individual calibration lines were noticeably different for each channel, ranging from 1.115 for channel 3 to 1.721 for channel 1 (Table 3). This resulted in a CV between the slopes of 18.5%.

When the experiment was repeated, substituting diazepam-d_5 for nitrazepam as the internal standard, the individual calibration curves for the four channels overlay directly. The slopes are identical, resulting in a CV of just 2% across the four channels (Table 3). This finding clearly demonstrates analyte-dependent differences in the responses for each channel and suggests that unless a deuterated internal standard is used, samples to be compared quantitatively must be analyzed through the same sprayer and each channel independently calibrated to afford true quantification.

This finding is perhaps not too surprising. One would expect close structural analogues (diazepam/diazepam-d_5) to have virtually identical mass spectrometric properties whereas more structurally diverse analytes (diazepam/nitrazepam and diazepam/clozapine as discussed previously) will behave differently. Additionally, as one would not expect all analytes to ionize identically when comparing separate ESI tandem mass spectrometer instruments, it may be predicted that analytes behave differently when analyzed via four completely separate capillaries comprising a MUX interface.

There have been two recently published applications of MUX analysis for quantitative bioanalysis.[37,38] The first literature example

Table 5. MUX Analysis of Rat Liver Microsomal Incubates[a]

parameter	incubation	channel 1	channel 2	channel 3	channel 4	mean ± SD	CV (%)
peak area diazepam	A	43 055	37 494	46 644	54 297	45 373 ± 7040	15.5
(−NADPH incubations)	B	43 617	38 340	46 458	54 104	45 630 ± 6575	14.4
	C	42 447	38 792	51 241	55 387	46 967 ± 7669	16.3
peak area diazepam	A	24 787	21 259	27 347	30 328	25 930 ± 3850	14.8
(+NADPH incubations)	B	25 909	23 522	28 992	32 396	27 704 ± 3846	13.9
	C	24 884	22 544	30 331	31 372	27 283 ± 4252	15.6
diazepam turnover (%)	A	42.4	43.3	41.4	44.1	42.8 ± 1.2	2.8
	B	40.6	38.6	37.6	40.1	39.2 ± 1.4	3.5
	C	41.4	41.9	40.8	43.4	41.9 ± 1.1	2.6

[a] Triplicate incubations (A−C) of diazepam (1 μM, 15-min incubations at 37 °C) in the presence and absence of cofactor NADPH (2 mM) were analyzed in triplicate through each channel of the MUX interface. The table shows average peak areas acquired for diazepam for both the plus and minus NADPH incubations and percentage turnover relative to the −NADPH control for each channel. For each incubate, interchannel CV is shown for both diazepam peak areas and overall diazepam percentage turnover for each MUX channel as calculated using the following formula: microsomal turnover (%) = 100 − ((peak area diazepam$_{(+NADPH)}$/peak area diazepam$_{(-NADPH)}$) × 100).

describes the use a multiplexed interface coupled to triple-quadrupole mass spectrometry for quantitative bioanalysis. Here, methodology for the direct, sensitive determination of a novel isoquinoline in plasma, with a throughput of 120 samples/h,[38] is presented. Excellent interchannel reproducibility for a series of calibration curves was achieved (CV <1%), and the stated conclusion is that a sample will yield the same analytical result irrespective of which column/sprayer it is analyzed on. However, the internal standard used in this work was the M + 6 deuterated isoquinoline analogue, which lends support to our findings. The second paper presents validation data for analytical methodology for the quantification of loratidine and its metabolite, descarboethoxyloratidine, in four matrixes using the MUX interface.[37] No mention of the issue of compound-dependent cross-channel differences in response is made, but by the very nature of the experimental design, each of the channels is independently calibrated using either rat, rabbit, mouse, or dog plasma, and as such, interspray variability issues are circumvented.

To establish the reproducibility and robustness of the MUX analysis, each calibration set was injected five times through each MUX channel. This produced five sequential calibration curves per channel. Reproducibility was demonstrated for each channel, and CVs for the five slopes obtained through each channel were ~10% for the diazepam/nitrazepam internal standard calibrations and up to 6% for the diazepam/diazepam-d_5 internal standard calibrations (Table 4).

The limit of quantification was 0.025 μM for each channel (7.1 ng/mL). This falls in to the generally accepted region required for higher throughput quantitative screening applications in a drug discovery environment, where the emphasis is on throughput of samples of many identities rather than ultimate sensitivity.

Application of MUX Analysis to Quantitative Bioanalysis. The availability of metabolism data in early drug discovery is now crucial to the decision-making process in lead optimization, and as such, there has been an increasing demand for higher throughput approaches to metabolic evaluation. As the liver represents the major organ for drug metabolism, one of the simplest and most commonly adopted approaches is to screen potential drug candidates for in vitro metabolic stability. This is commonly achieved by incubating these compounds with hepatic preparations, in particular, microsomal fractions.[42] Here, single-concentration, single-time point incubations are generally performed. Stability assessments are then made by analysis of the incubates, usually by mass spectrometry, for disappearance of test compound.[43] Traditional methods for the investigation of in vitro metabolism employ manual, labor-intensive techniques. These require relatively large volume incubations in vials carried out in shaking water baths. However, with the advent of robotics, many of these time-consuming processes have been replaced with more automated approaches where incubations in microtiter plate format are performed using robotic liquid handling. As a consequence, sample preparation is no longer the rate-determining step and higher throughput approaches to sample analysis are required.

In the current investigation, in order to provide large enough samples for multiple repeat analyses, 2-mL triplicate incubations of diazepam (1 μM) in rat liver microsomes were performed under oxidative conditions in vials in a shaking water bath. Triplicate control incubations were performed in the absence of cofactor. Each incubation sample was analyzed in triplicate through each

Table 6. Robustness of MUX Interface Following Multiple Injections of Biological Samples (Rat Liver Microsome Incubates) with HPLC Solvent-Front Flow Diversion To Prevent Source Fouling[a]

channel	peak area diazepam (prior to injection of biological samples) mean ± SD	CV (%)	peak area diazepam (following injection of biological samples) mean ± SD	CV (%)	rel resp (%)
1	20 344 ± 1529	7.5	19 578 ± 2159	11.0	96.2
2	20 087 ± 826	4.1	19 429 ± 712	3.7	96.7
3	27 086 ± 1624	6.0	26 117 ±1359	5.2	96.4
4	24 229 ± 920	3.8	23 849 ± 791	3.3	98.4

[a] Peak areas ± SD obtained for repeat injections of a diazepam standard solution (0.5 μM, n = 24 injections/channel) prior to and following multiple injections of microsomal incubates (n = 48 injections/channel).

(42) Masimirembwa, C. M.; Thompson, R.; Andersson, T. B. *Comb. Chem. High Throughput Screening* **2001**, *4* (3), 245−263.
(43) Korfmacher, W. A.; Palmer, C. A.; Nardo, C.; Dunn-Meynell, K.; Grotz, D.; Cox, K.; Lin, C.-C.; Elicone, C.; Liu, C.; Duchoslav *Rapid Commun. Mass Spectrom.* **1999**, *13* (10), 901−907.

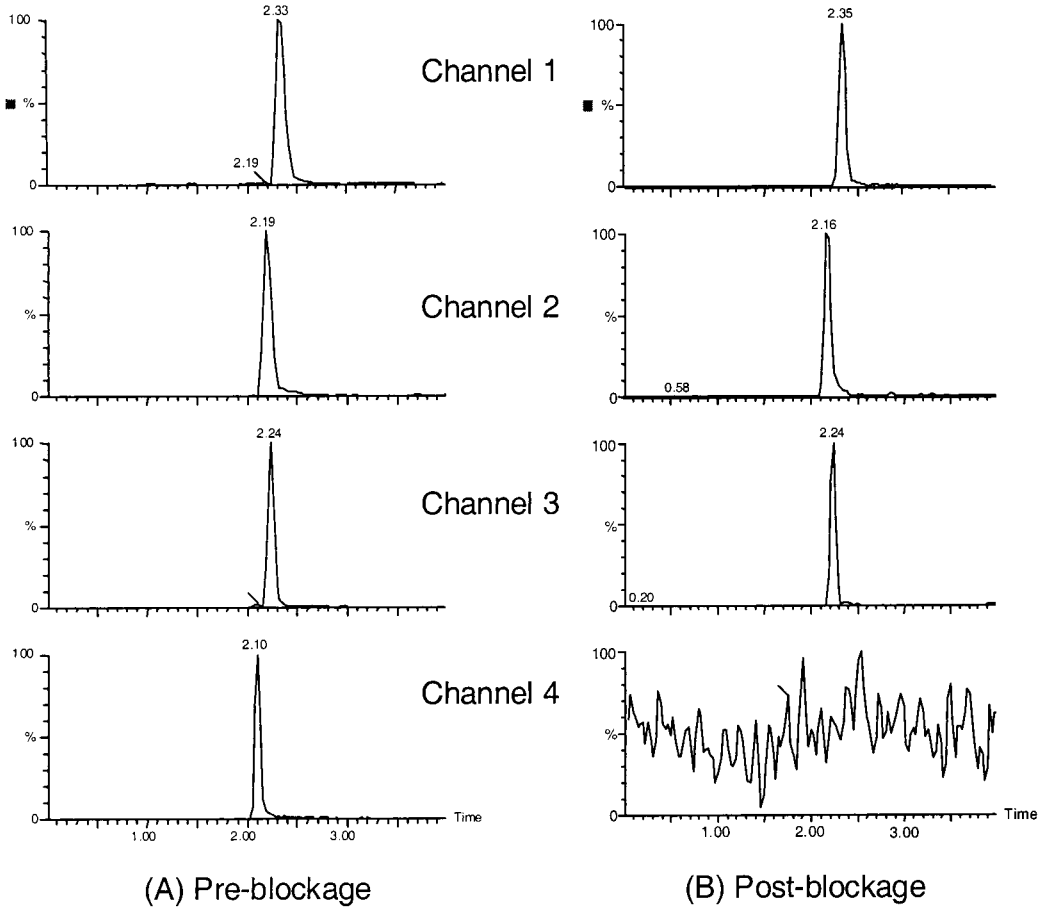

Figure 1. Multiple reaction monitoring ion chromatograms acquired for diazepam (0.5 μM) by analysis via four channels of the MUX interface showing the value of individually back-pressure-regulated HPLC pumping systems for each column. Here, blockage of a single channel (channel 4) occurred with no resulting detrimental effects on the chromatography for the remaining three channels.

channel of the MUX interface. The peak areas acquired for diazepam for both the plus and minus NADPH incubations, when analyzed through each channel, demonstrated good reproducibility. However, as previously shown, channel differences in response were observed in that identical samples injected across channels showed variation in peak area. Typical interchannel coefficient of variation was 15% (Table 5). Nevertheless, once one calculates the percentage turnover of diazepam, which is based on the ratio of the peak areas obtained in the plus and minus NADPH incubations injected via the same channel, excellent cross-channel reproducibility is demonstrated. In this case, for the turnover results, an interchannel CV of ~3% was obtained. This finding is in good agreement with the previously stated argument that samples for quantitative comparison need to be analyzed through the same channel/sprayer unless a deuterated internal standard is included.

Additionally, it was found that when multiple replicate injections of a 0.5 μM diazepam stock solution ($n = 24$ injections/channel) were made both immediately prior and postinjection of multiple biological incubates, no deterioration in peak area response or reproducibility was observed for these standards (Table 6). This demonstrates that using the current instrument configuration with flow diversion for the first minute of each analytical run causes no source fouling upon injection of biological samples. As a result of this, no detrimental effects on instrument performance are experienced.

Advantages and Disadvantages of MUX Analysis. Aside from the obvious advantage of increased analytical throughput, there are general noteworthy discussion points of the multiplexed electrospray inlet system approach to parallel mass spectrometric analysis. One feature of the described system is the advantage of individually back-pressure-regulated pumping channels. This is an advantage over previously reported systems that rely solely on flow splitting by virtue of simple "T-piece" splitters which require absolute care to ensure that the back pressures in all four channels are kept the same.[33,37] With the system described herein, column flow rate is not simply a function of column back pressure, so if one channel were to become blocked, the flow through this channel is merely diverted to waste. The benefit of this is that there is no effect on the chromatographic performance of the remaining three channels (Figure 1) or the reproducibility of response (data not shown). This is an important point since it would be highly undesirable, when employing MUX analysis, to lose multiple samples because of a single channel failure. This scenario would not occur in the case of a single ESI channel interface instrument where a high- or low-pressure error would result in automatic HPLC shutdown and no loss of precious samples.

One perceived problem of this parallel analysis approach is the possibility of interchannel cross-talk. This was previously reported as negligible,[35,37,38] and it is our belief that cross-talk interferences are unlikely to be a problem as the applications that

we feel are most suited to MUX analysis are bioanalytical screening procedures. Here, analyses of multiple test compounds with just a few samples (<10) per compound are required. As different analytes will be injected through each sprayer interchannel, cross-talk will not be an issue.

A key observation made in this investigation was that it is crucial to ensure that the maximum total flow into the MUX interface does not exceed 400 μL/min, i.e., 100 μL/min per channel. Incomplete desolvation can often result in poor response reproducibility. However, by combining a postcolumn eluent split for each of the four sprayers with the use of independently back-pressure-regulated pumping systems, the ratio of flow to source to waste should remain constant and source flooding will not present a problem.

CONCLUSIONS

Our findings suggest that the multiplexed interface approach allowing the parallel MS/MS analysis of multiple samples can be employed to increase the bioanalytical throughput in a typical drug discovery environment. We have demonstrated that, although the overall detector sensitivity is somewhat reduced compared with conventional single ESI interfaces, suitable detection limits and response linearities are achievable with MUX analysis, with the obvious increase in sample throughput. The use of generic HPLC gradients and the parallel analysis of four samples means that very little experimental optimization is required apart from MRM transition determination, for which automated software packages are available.

However, we have shown that analyte-dependent differences between MUX sprayers do exist, so samples for quantitative comparison require analysis via the same channel. Alternatively, if a deuterated internal standard is available, which is generally not the case in a drug discovery environment, samples may be analyzed across all four channels. Furthermore, with the autosampler cherry-picking capabilities of the described system, it is possible to simply program injector sequences to comply with these criteria.

It is recommended that MUX technology is applied for the routine MS/MS analysis of drugs in biological media in support of higher throughput DMPK screening procedures. It is anticipated that, by adopting such an approach, it will become more feasible to keep pace with the increased sample numbers achievable with recent advances in automated sample preparation techniques, such that sample analysis is no longer the rate-determining step.

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