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A new approach to the application of solid phase extraction disks with LC–MS/MS for the analysis of drugs on a 96-well plate format

Erasmus Cudjoe, Janusz Pawliszyn*

Department of Chemistry, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, N2L 3G1 Canada

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ABSTRACT

A new 96-well disk solid phase extraction sample preparation technique which does not involve vacuum pumps integrated with liquid chromatographic tandem mass spectrometric (LC–MS/MS) was developed for high throughput determination of benzodiazepines (nordiazepam, diazepam, lorazepam and oxazepam). In addition, the method completely allows the re-use of the SPE disk membranes for subsequent analyses after re-conditioning. The method utilizes a robotic autosampler for parallel extractions in a 96-well plate format. Results have been presented for independent extractions from three matrices; phosphate buffer solution, urine, and plasma. Factors affecting data reproducibility, extraction kinetics, sample throughput, and reliability of the system were investigated and optimized. A total time required per sample was 0.94 min using 96-well format. Method reproducibility was ≤9% relative standard deviation for all three matrices. Limits of detection and quantitation recorded were respectively in the range 0.02–0.15 and 0.2–2.0 ng/mL with linearity ranging from 0.2 to 500 ng/mL for all matrices.

1. Introduction

Typically quantitative analyses of drugs in biofluids have been achieved with liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) by most drug industries. The primary reason is that the effectiveness of the separation power of liquid chromatography (LC) and the ultra-low sensitivity, selectivity and identification offered by mass spectrometer have significantly paved the way for the development of improved bioanalytical methods [1]. Moreover, in bioanalysis, most drugs are not compatible with gas chromatographic separations and analysis, unless derivatization to increase volatility is performed which adds another step to the analytical process.

Notwithstanding the success of the LC–MS/MS technique in drug discovery, most of the traditional bioanalytical methods require extensive offline sample preparation methods which are often carried out manually [2]. This in part has led to the desire for faster bioanalytical sample preparation methods with low limits of detection (LODs).

In recent times, the efficiency of a sample preparation method is not only measured by its precision, sensitivity and accuracy. In addition to these important parameters, an ideal sample preparation methodology must include high sample throughput; consume minimal amount of solvent; be simple, safe and cost-effective and in some cases, be amenable to automation. Some of the conventional sample preparation methods like liquid–liquid extraction (LLE) and solid–liquid extraction (SLE) suffer major setbacks due to their low sample throughput, large volumes of solvent required, cost of solvent disposal methods and environmental safety issues [3,4]. In addition, both methods are not easily automated. Owing to some of these setbacks, the conventional methods have in part given way to other sample preparation methods like solid phase extraction (SPE), solid phase microextraction (SPME), headspace extraction (HSE), and purge and trap (PT), which to a large extent address these setbacks.

For drug analysis in biological matrices, SPE method in which analytes are exhaustively extracted from solution into a sufficient volume of an extraction phase has gain tremendous popularity. This might primarily be due to the pre-concentration of the analytes from the matrix and the advantage of automation to increase sample throughput. Generally, SPE sample preparation method employs chromatographic stationary phase which efficiently extracts targeted analytes from complex matrices [5] after which small solvent volumes can be used to elute the trapped analytes. Various separation techniques like the Sep Pak C_{18} [6–8], Bond Elut Certify [9], Isolute C_2 [10], to mention a few have been used for drug analysis. However, the disk SPE technology which forms the second generation of SPE methods since its introduction in the 1990s [11] has also extensively been used for the bioanalysis compared to the conventional SPE cartridges or columns. Various benefits of the disk SPE techniques for sample preparation have been well outlined [12,13].

* Corresponding author. Tel.: +1 519 888 4567x84641; fax: +1 519 746 0453.
E-mail address: janusz@uwaterloo.ca (J. Pawliszyn).

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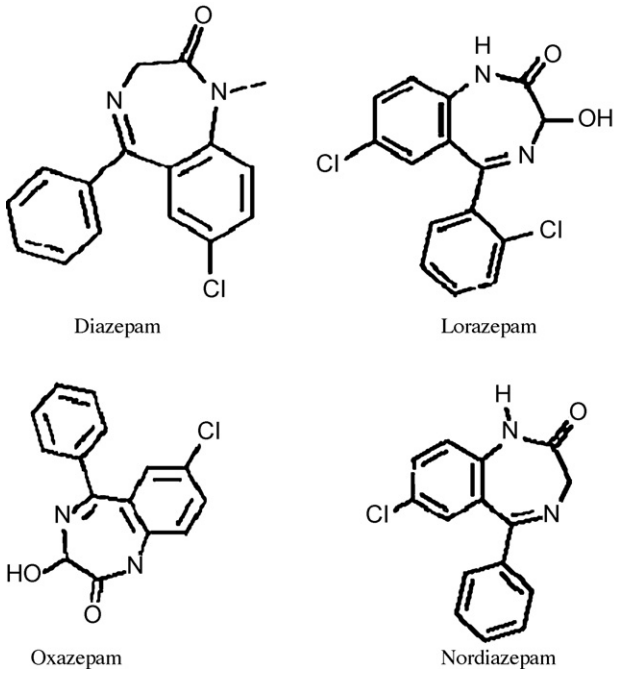

Fig. 1. Molecular structure of the selected benzodiazepines.

The disk SPE material is a thin membrane disk (0.5–0.75 mm in thickness) made up of polytetrafluoroethylene (PTFE) or glass fibre saturated with fine bonded silica that decreases the channelling and/or clogging commonly associated with extraction from biological matrices like plasma or serum [14]. A number of articles concerning the analysis of drugs in biological matrices using disk SPE methods have been published [15–18].

Apart from been automated, in order to further improve sample throughput, SPE has also been used on a multi-well plate format. Matthews et al. demonstrated the ability to extract rofecoxib from human plasma using SPE on a 96-well plate format [19,20]. In 2001, Biddlecombe and his group demonstrated the potential of SPE sample preparation method for bioassays on a 384-well plate format [21]. Simpson et al. also demonstrated the use of disk SPE method for high throughput LC–MS/MS bioanalysis on a 96-well plate format [22]. Despite the fact that disk SPE automation and its application on a multi-well pate improves throughput compared to the conventional solid phase extraction method, the setup could be complicated with many cartridges and valves, expensive and also requires the use of effective vacuum pumps.

The aim of this paper is to introduce the use of a simple integrated and quantitative disk SPE sample preparation method capable of performing parallel extraction of drugs on a 96-well plate format. The coupling of disk SPE to multi-well plate format was accomplished using a robotic autosampler but avoiding the use of pumps. The proposed, simple clean-up method also allows the re-use of the thin disk membranes, thus reducing cost of analysis per sample. The drugs chosen for this project are benzodiazepines (diazepam, nordiazepam, oxazepam and lorazepam; Fig. 1) and were selected for their known varying sedative, anxiolytic, anticonvulsant, muscle relaxant and amnesic properties.

2. Experimental

2.1. Chemicals and materials

HPLC grade methanol and acetonitrile solvents used in the experiments were obtained from EMD Chemicals Inc. (Darmstadt, Germany). Acetic acid was obtained from BDH Inc. (Toronto, Ontario). High performance SPE extraction disks were purchased from 3M (Empore™) (St. Paul, Minnesota, United States). The benzodiazepines (diazepam; nordiazepam; oxazepam; and lorazepam) each of concentration 1 mg/mL in methanol with the exception of lorazepam, which was in acetonitrile, were obtained from Radian International (Austin, TX, United States) and stored at 4 °C in a refrigerator. A mixed standard (1 ng/mL) of the benzodiazepines was prepared in 1:1 (v/v) methanol–water mixture, stored in the fridge, and used as the stock solution for subsequent experiments. Deionized water used for dilution of stock solutions was from a Barnstead/Thermodyne NANO-pure ultra water system (Dubuque, IA, United States). Drug-free urine and blood samples were obtained from a healthy volunteer.

2.2. Instrumentation, HPLC and mass spectrometry conditions

A Shimadzu® (LC-10 AD) HPLC coupled with a tandem triple quadrupole mass spectrometer (MS) (API 3000, Toronto, Canada) was used for separation and analyses of the benzodiazepines. All extracted analytes were injected into the Shimadzu® HPLC coupled to the API 3000 MS using the CTC PAL auto-injector from Leap Technologies (CTC Analytics, Carrboro, NC, United States). Chromatographic separation was achieved with gradient elution using an acetonitrile–water mixture as mobile phase. Mobile phase A was 10:90 (v/v) while that of B was 90:10 (v/v) acetonitrile–water mixture with 0.1% acetic acid in both mobile phases to enhance ionization. A sample volume of 20 μL was injected, with a flow rate of 0.5 mL/min maintained during the 5 min chromatographic separation. In the case of the spiked biological samples (urine and plasma), a TosoHass TSK precision bypass pump, ran in isocratic mode (flow rate 0.5 mL/min) and Waters® switching valve were used to direct column effluent in the first minute of chromatographic process from the MS.

The API 3000 triple quadrupole MS, equipped with a TurboIonSpray Source and heated pneumatic nebulizer interface was operated in the positive mode under multiple reaction monitoring (MRM) conditions. The source temperature and voltage were set at 250 °C and 4500 V, respectively. The curtain, nebulizer, and collision gases were also set at 10, 6, and 12, respectively. The Q1 and Q3 MS acquisition parameters for each drug were given in Table 1. Data analysis was performed using Analyst 1.4.1 software which is integrated with the API 3000 instrument.

2.3. Preparation and conditioning of extraction materials

The extraction disks used for this project were silica sorbents with octadecyl group attached to the silica surface. This implies that the disk would demonstrate a greater potential for retaining non-polar analytes. It had a 22.5% carbon loading, 60 Å pore size and average particle size of 12 μm. The SPE disks were first evaluated for their suitability to extract the targeted drugs and secondly for use on a 96-well format. To ensure same surface area to volume ratio, the disks were cut into equal smaller sizes of 5 mm diameter using a paper-hole puncher. The smaller size disks were suspended at the tip of the stainless steel pins as illustrated in Fig. 2. This was done

Table 1
MS acquisition parameters

Drug	Q1 mass	Q3 mass	DP	FP	EP	CE	CXP
Diazepam	285.0	153.9	92	120	7.5	39	10
Nordiazepam	271.1	140.0	66	170	10	39	10
Oxazepam	287.1	241.1	61	160	10	31	18
Lorazepam	321.1	275.1	101	101	10	31	20

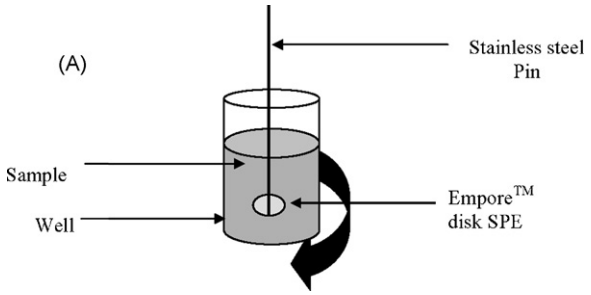

Fig. 2. Schematic diagram showing SPE disks used as extraction phase supported at the tip of a stainless steel pin.

by piercing through the smaller disks with the stainless steel pins. An in-house multi-fibre support (Fig. 3) designed to fit into a 96-well plate was used to anchor the pins with the suspended disks in place such that each pin fits into a well in the 96-well plate (Fig. 4). By this configuration, each of the 96 disks was well centred in each of the well in the 96-well plate. Prior to each set of experiments, the membranes were conditioned with 1 mL of methylene chloride followed by 1:1 methanol:water (v/v) and then agitated at 850 rpm for 10 min.

2.4. Automated disk SPE method for LC–MS/MS on a 96-well plate format

To be able to perform parallel disk extraction of 96 samples, an automated robotic unit (Concept 96) was obtained from Professional Analytical System Technology (PAS, Magdala, Germany). The fully automated robotic unit consisted of three integrated parts and separate two orbital agitators and was controlled with Concept® software. One of the parts designed to hold, move, and place the extraction phase into the 96 wells was used primarily for the extraction and desorption processes while agitating the well-plate at a specific speed. The second part was designed to be used for solvent reconstitution and/or analyte pre-concentration steps for situations were enhanced sensitivity was required. This part equipped with an evaporation device allows the flow through of nitrogen gas. The device was setup just above each well which allows direct flow of nitrogen gas into the well to evaporate the solvent. The third part of the robotic unit performed a dual role of dispensing specific volumes of solvents into the individual wells of the 96-well plate in addition to the injection of samples into the HPLC port for chromatographic separation. The dispensed solvents could be used as desorption, reconstitution and/or internal standard solvents. However, for the sake of this project this feature was not used since all samples and solvents were pipetted manually with no need for vacuum pumps.

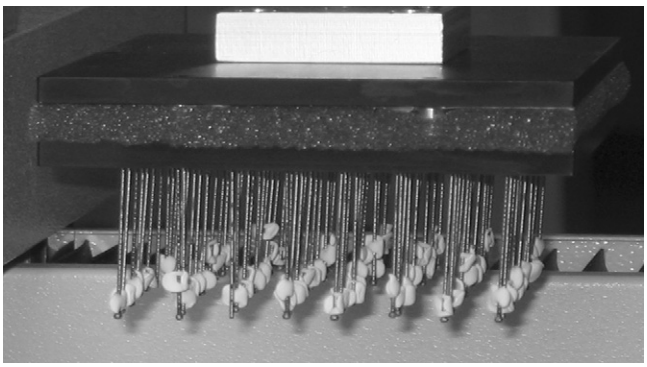

Fig. 4. 96 thin films made from Empore™ disks SPE suspended on stainless steel pins and supported on an in-house multi-fibre holder.

Combined with the two agitators, the above configuration of the automated robotic unit allowed for complete automation of the entire disk SPE sample preparation procedure on a 96-well plate format with the exception of transfer of sample and solvents where manual pipettes were used. More information on the use of the PAS robotic autosampler for other sample preparation technique can be obtained elsewhere [23].

2.5. Preparation of calibration standards

Stock solutions of the four analytes originally kept in the refrigerator at 4 °C were serially diluted in 1:1 (v/v) methanol–water to known concentrations and used as stock for further analysis. Specific amounts of the stock were added to each matrix while maintaining the volume of organic solvent constant in each case to generate a set of calibration standards (0.2–500 ng/mL). Blank samples were used to check for any interferences and instrument performance.

2.6. Experimental procedure

Fixed volume (1 mL) of samples were placed inside the wells and the disks were immersed into the samples for varying times for the analytes to be trapped on the disks from the matrix. This was done to ensure that optimum amount of the analytes would be extracted in order to improve the extraction capacity and thus sensitivity. After the extraction process, the analytes were desorbed from the disks by using optimized solvent system for which the analytes had the greatest affinity. After establishing the extraction capability of the disks, method optimization was carried out using 1 mL of 50 ng/mL benzodiazepines standard in phosphate buffer solution maintained at pH 7.4. The method optimization processes included the determination of agitation speed which would improve kinetics

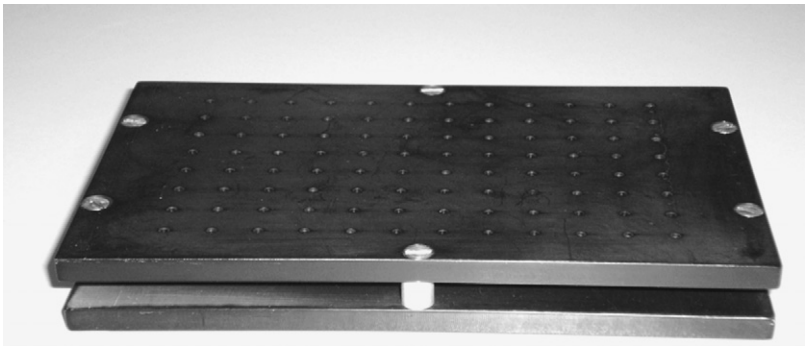

Fig. 3. An in-house multi-fibre support designed for a 96-well plate.

Table 2
Optimized sample preparation conditions for analysis of benzodiazepines

Parameter	3M Empore™ disk SPE
Agitation speed	850 rpm
Desorption solvent	50% methanol solution
Equilibration time	40 min
Desorption time	30 min

of analyte transfer in the system, extraction and desorption times, disk washing step and the composition of desorption solvent and finally the amount of each analyte carried over from previous experiments. After optimization process, the method was applied to the extraction of the benzodiazepine drugs from PBS buffer, urine and plasma. Table 2 shows the optimized sample preparation conditions used for the analysis of benzodiazepines in PBS buffer, urine and plasma.

All samples used were separately made to 50 ng/mL of benzodiazepines by spiking the sample matrix with known amount of each drug. The spiked drug samples were allowed to equilibrate within matrix for 12 h. Inter- and intra-well variations for the 96-well plate were determined including repeatability and percent recoveries from each matrix. With the exception of the optimization experiments in which sample were placed in selected wells, parallel sample preparations were completed for 96 samples using the automated robotic unit from PAS.

3. Results and discussion

3.1. Stability of standards and assay

The stability of each analyte during analysis was determined by comparing instrument response of freshly prepared standard solution before and after each analysis with previous standard solution. In addition, post-preparative stabilities were determined after a 30-day period of storage after which new standards were prepared from the stock although the analytes were found to be very stable within the period of storage. In the case of PBS and urine assays, stabilities were determined for three replicates of 50 ng/mL of each analyte after a 24-h period after the spiked samples were left under ambient conditions for 12 h.

Analyses of three blank samples of all three matrices did not reveal any interference from the chromatographic runs.

3.2. Development of sample preparation method

In recent times, development of bioanalytical methods for high throughput analysis cannot be overemphasized. Owing to the fact that SPE, especially methods developed using membrane disks, continues to demonstrate remarkable potential for the analysis of drugs in biological samples, our method focused on using disk SPE for parallel analyses of drugs on a 96-well format. The choice for using Empore™ C_{18} extraction disks was firstly due to their non-polar characteristics which is similar to the targeted analytes and secondly the shorter equilibration time over that of polydimethylsiloxane (PDMS) coating determined elsewhere [23]. This implies that, the use of disk SPE membranes would offer shorter analysis time and thus higher sample throughput for parallel analyses on a 96-well plate.

Some of the advantages of the technique were that the need to buy more expensive 96-well disk SPE cartridge is completely avoided by suspending smaller size (5 mm) disks obtained from the original 47 mm diameter disk. In addition, the in-house multi-fibre holder or the pin-tool replicator used can be easily controlled manually without robotic autosampler as demonstrated by Hutchison et al. in the analysis of PAHs in environmental samples [24] although that compromises throughput due to manual replacement of the 96-well plates. Another advantage of this extraction technique is the fact that the disk membranes are re-usable provided the disks were re-conditioned and also the method does not require the use of vacuum pumps, thus making it cost-effective.

3.3. Agitation speed

Although higher agitation speeds could be used, which would in turn lead to faster extraction and desorption times and thus increase sample throughput, inter-well sample or solvent contaminations occurred at higher agitation speeds due to spilling from some of the wells. As a result of this limitation, effective kinetics of diffusion and mass transfer was achieved with the two agitators at an optimum agitation speed of 850 rpm which was used for all the subsequent experiments. Alternatively, smaller sample volumes could be used with higher agitation speeds.

3.4. Extraction and desorption time profiles

With an agitation speed set at 850 rpm, the equilibration time for diazepam, nordiazepam, oxazepam, and lorazepam was found to be 40 min as shown in Fig. 5. However, a 60 min extraction time was selected for this project. This precautionary measure was adopted primarily because for effective mass transfer during the extraction and desorption processes, the position of the extraction material inside the sample contained in the well is very pertinent. Thus by increasing the extraction time, the extent of error that may occur due to the movement of some of the thin films suspended on the stainless steel pin inside the well during agitation would be significantly minimized.

Best results, in terms of possible solvent spillage from wells during extraction (cross contamination), desorption time and amount carryover, were obtained with 1:1 (v/v) methanol–water after testing other solvent systems like pure methanol and acetonitrile, and 10% methanol–water. Acetonitrile and 10% methanol–water were eliminated due to significant cross contamination and carryover, respectively.

Optimum desorption condition was therefore determined by comparing the desorption efficiencies of 1:1 (v/v) methanol–water and pure methanol with respect to time and the amount of analyte carried over. From the results, notwithstanding the fact that methanol was a better desorption solvent and offers shorter desorption time (25 min), 1:1 (v/v) methanol–water (Fig. 6) was used in this study because of solvent spill over, which results from higher agitation speeds. Another advantage was that the use of 1:1 (v/v) methanol–water desorption solution would reduced the rate of possible evaporation that may introduce inherent errors when longer desorption times were used. To ascertain this assumption, the relative standard deviation (R.S.D.) of the

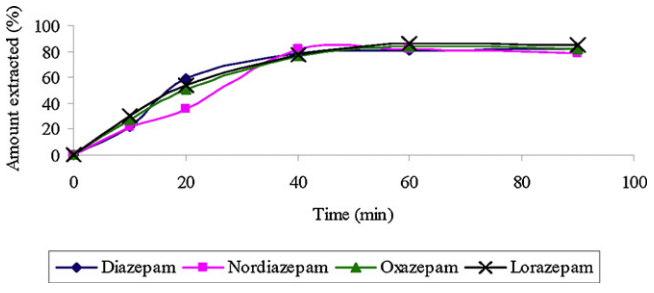

Fig. 5. Extraction profile for benzodiazepine drugs using 3M Empore™ disk SPE.

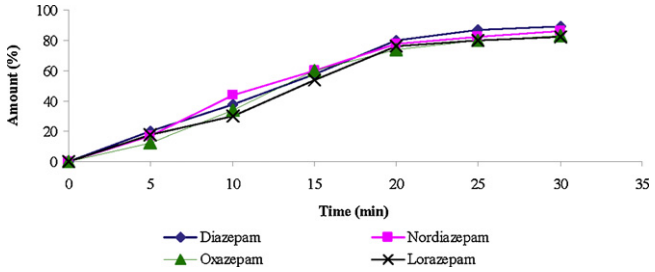

Fig. 6. Desorption profile for benzodiazepine drugs using 3M Empore™ disk SPE in a 1:1 (v/v) methanol–water.

amount of analyte extracted for each compound at 25 min, using 1:1 (v/v) methanol–water and pure methanol were determined. Results showed lower R.S.D. values when a 1:1 (v/v) methanol–water (2.9–5.2%) solution was used compared with pure methanol (5.0–8.3%). It was therefore conclusive that 1:1 (v/v) methanol–water desorption system would provide more reproducible data with less solvent evaporation.

As part of the optimization process, subsequent to each desorption process carry over experiments were performed to investigate the amount of each of the benzodiazepine carried over from previous desorption process. This was monitored to prevent introduction errors into subsequent experiments due to incomplete analyte desorption from the thin films. From the carry over experiments, nearly complete desorption of the benzodiazepines from the thin films in methanol and 1:1 (v/v) methanol–water solvent systems was achieved at 25 and 30 min, respectively. All the analytes had ≤0.3% relative amount carried over when desorbed in 1:1 (v/v) methanol–water solvent system for 30 min. No carry over was detected for diazepam and lorazepam while nordiazepam and oxazepam were ≤0.3 and 0.2%, respectively. In addition, the reconditioning of the disk prior to each analysis completely removes all the analytes making it re-usable. Fig. 7 shows the plot of amount of analyte carryover after each desorption step using 1:1 (v/v) methanol–water.

3.5. Effect of pre-washing of thin films

In most extractions from biological samples, it is relevant to investigate the effect of the sample matrix since the presence of non-specifically bound interfering compounds may significantly affect results. Therefore, investigation was carried out to compare the amount of benzodiazepines extracted from spiked urine and plasma samples in the presence and absence of pre-washing of thin films prior to desorption. From the results, inter-well variations for three replicate extractions were determined for selected representative wells. As shown in Table 3, there were no significant variations for sample preparation method completed with/without pre-washing of the thin films. Absolute recoveries calculated for each matrix were comparable which reflects the analyte uptake was not affected.

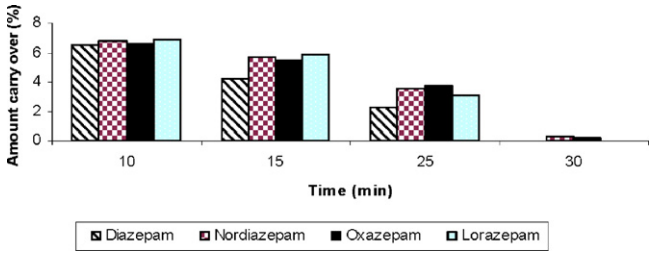

Fig. 7. Amount of analyte carried over at various desorption times using 1:1 (v/v) methanol–water mixture.

Table 3
Comparison of extraction efficiencies and inter-well variations in the absence and presence of pre-washing of Empore™ disk SPE ($n = 3$)

Drug	Pre-washing		No Pre-washing	
	Urine	Plasma	Urine	Plasma
R.S.D. (%)				
Diazepam	6.3	6.7	6.1	6.7
Nordiazepam	6.9	7.3	6.9	7.4
Oxazepam	8.1	8.5	7.9	8.6
Lorazepam	7.9	9.1	8.5	8.9
Absolute recoveries (%)				
Diazepam	84	79	85	80
Nordiazepam	83	79	81	80
Oxazepam	79	75	77	72
Lorazepam	75	72	77	73

3.6. Reproducibility of extractions using Empore™ disk and re-usability

One of the important characteristics of every analytical sample preparation method, apart from data accuracy, is the reproducibility of the data. This factor becomes very important for parallel extractions due to variations that might occur between and within the wells for each sample preparation. It also offers an opportunity to ascertain the reliability of the PAS robotic automated system used for the project.

Inter-well variations were investigated by calculating the overall percent relative standard deviation (R.S.D.%) for all the 96 wells for each analyte. The mean amount extracted from all 96 wells and standard deviation was used to determine the R.S.D.% for each compound. Table 4 shows a summary of inter-well R.S.D.% obtained for all the analytes extracted from the various matrices. For the PBS saline buffer extractions, the percent R.S.D.s ($n = 96$) were ≤6% for five successive-independent extractions. With the exception of oxazepam and lorazepam that gave relatively higher R.S.D.s, diazepam and nordiazepam were generally ≤5% for all the five separate extractions (data not shown). For urine and plasma samples, the range for R.S.D.% falls within 6–9% which was only slightly higher than that for PBS buffer. From the results, despite the movement of the disk membranes inside the wells, a steady agitation was achieved under the set experimental conditions and that variability between the disk membranes was within acceptable levels.

The reproducibility of the amount extracted from the same well (intra-well variations of the benzodiazepines extracted) were also calculated for the five successive extractions. The percent R.S.D.s obtained for intra-well variations for the benzodiazepines were in the range 2–13%. Fig. 8 shows the intra-well variations obtained for five-independent extractions of all the drugs from all 96 wells. Minor variations that occurred from successive extractions may primarily be attributed to the movement of the disk membranes inside the wells since they were not rigidly attached to the stainless steel pins. Thus, for some of the experiments the relative positions of the

Table 4
Summary of results obtained for inter- and intra-well variations and the corresponding absolute recoveries for the benzodiazepines in three different matrices ($n = 5$)

Sample matrix	Inter-well R.S.D. (%)	Intra-well R.S.D. (%)	Recovery (%)
PBS	4–6	3–9	82–89
Urine	6–8	2–11	76–88
Plasma	6–9	3–13	73–84

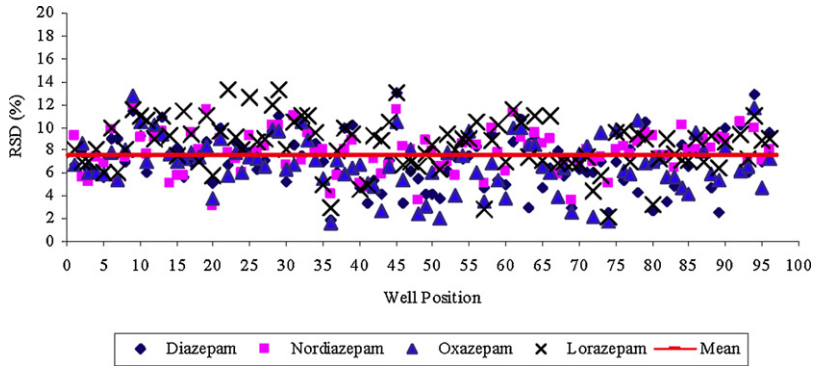

Fig. 8. Intra-well variations for five successive extractions of benzodiazepines from urine with the Empore™ disk SPE.

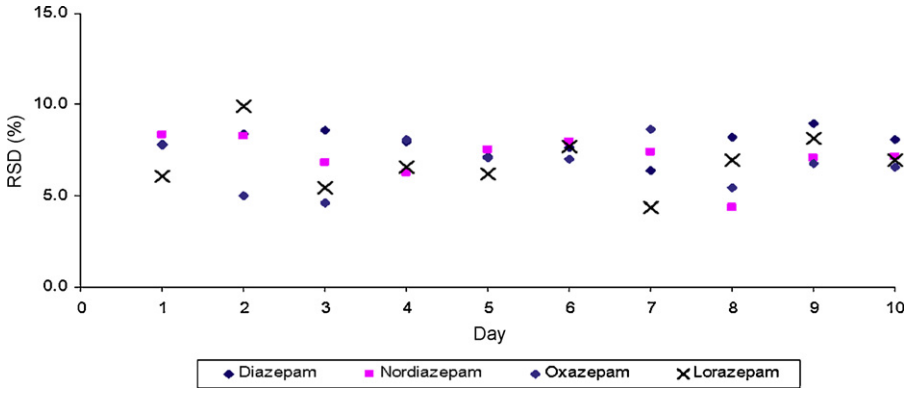

Fig. 9. Inter-day variations for extractions of benzodiazepines from PBS buffer using Empore™ disk SPE.

disk membranes in the wells were altered during each extraction. For example, in the case of well position 91, it was observed that the disk membrane during each extraction moves to the surface of the sample solution throughout the extraction and desorption processes. This resulted in the extracting phase not having adequate physical contact with the sample solution for effective mass transfer during agitation. The R.S.D.s consequently were considered as outliers in this study. This finding indicates that during agitation, having a fixed position of the extracting material was very crucial to achieving more reproducible data. Despite this minor setback, the R.S.D.s obtained fell within acceptable limits for bioanalytical purposes, which affirm method reproducibility for the analysis of benzodiazepines (Fig. 8).

Fig. 9 shows the precision results for the extractions of the benzodiazepines from PBS buffer on different days using the same set of disk membranes ensuring that the disk membranes were re-condition prior to each experiment. The analyses were done for selected number of wells with three-independent extractions performed each day. The R.S.D.% calculated which ranged from 4 to 11% showed excellent method reproducibility and in addition affirms the fact that the disk membranes were re-usable.

3.7. Detection and quantitation limits and linear range

Limit of detection (LOD) was determined based on the signal to noise ratio (S/N) method as 3× S/N for all three matrices. The LODs ranged from 0.02 to 0.15 ng/mL for all the benzodiazepines in the three matrices with lorazepam been the highest of the four analytes. There was a minimal increase in the LODs as the matrix changes from PBS to urine and subsequently to plasma. This implies that the disk membranes had very high affinity for the analytes and thus totally extracts the analytes from the various matrices. Limits of quantitation which ranged from 0.2 to 2.0 ng/mL for all the analytes in the different matrices was determined as 10× S/N. The method had excellent linear range which fell within 0.2–500 ng/mL for all three matrices. Calibration curves constructed from plots of peak area versus standard concentration yielded coefficients of regression typically greater than 0.9886 for all three matrices.

3.8. Assay throughput

From the results, a total time of 90 min was used for the analysis of 96 samples although the sample preparation time could be performed in 70 min. This implies that sample preparation time required per sample is less than 1 min, which makes it a potential alternative to other existing methods developed on a 96-well format for *in vitro* batch drug analysis.

4. Conclusion

The overall data affirm excellent performance of the disk SPE method for the automated analysis of drugs on a 96-well plate format. Despite the minor drawbacks due to the movement of the extraction disks inside the wells, the application of a simple integrated 96-well disk SPE technique in combination with a total chromatographic and analysis time of 5 min also shows another potential alternative the method with high sample throughput for bioanalysis. In addition, the technique demonstrated the potential of avoiding the use of vacuum pumps, re-usability of extraction materials and purchase of expensive 96-well disk SPE cartridges making it more cost-effective. This is very important because with an improved system where the extraction phase can be immobilized onto a surface, such as stainless steel plates, reproducibility can be significantly enhanced and also methods with shorter

extraction times could be developed for different extraction materials. In addition, the method provides the stage for exploring various extraction phases tailored toward *in vitro* batch bioanalysis.

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