Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 739–749
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

A novel incubation direct injection LC/MS/MS technique for in vitro drug metabolism screening studies involving the CYP 2D6 and the CYP 3A4 isozymes

S. Bhoopathy[a], B. Xin[b], S.E. Unger[b], H.T. Karnes[a,*]

[a] *Department of Pharmaceutics, Medical College of Virginia, Virginia Commonwealth University, P.O. Box 980533, Richmond, VA 23298-0533, USA*
[b] *DuPont Pharmaceuticals Company, Drug Metabolism and Pharmacokinetics, Stine-Haskell Research Center, 1090 Elkton Road, P.O. Box 30, Newark, DE 19714-0030, USA*

Received 16 August 2004; accepted 27 August 2004
Available online 30 December 2004

Abstract

A direct injection LC/MS/MS method involving a novel incubation technique was developed for the inhibition screening of CYP 2D6 and CYP 3A4 isoenzymes using dextromethorphan and midazolam as probe substrates. Both assays were performed using an electrospray ionization source in the positive ion mode. Direct injection was possible by using a short C 18, LC column (2 mm × 20 mm) with large particle diameter packing (10 μm). Analytical characteristics of the direct injection technique were studied by examining matrix effects, which showed suppression of the ESI signal between 0.20 and 0.65 min. The retention times for analytes were adjusted to approximately 0.8 min ($k' > 3$), resulting in no matrix effect. Column lifetime was evaluated and determined to be approximately 160 direct injections of the matrix. The precision and accuracy of the control samples for the quantitation of dextromethorphan was between −0.53 and −12.80, and 3.73 and 6.69% respectively. Unlike conventional incubation techniques, incubations were carried out in an autosampler equipped with a heating accessory. This novel incubation method, which involved no stirring of the incubation mixture, estimated the $Cl_{int\,in\,vitro}$ for dextromethorphan and midazolam in human liver microsomes to be 1.65 ± 0.22 ml/(h mg) and 0.861 ml/(min mg) respectively. The autosampler tray maintained uniform temperature and was sensitive to changes in temperature between 33 and 41 °C. High-throughput screening was performed using known inhibitors of the CYP 2D6 isozyme, and the system was evaluated for its ability to differentiate between these inhibitors. The strong inhibitor quinidine resulted in a 25.6% increase in $t_{1/2}$, the medium potency inhibitor chlorpromazine resulted in an increase of 6.14% and the weak inhibitor primaquine had no significant effect on half-life. This technique involves no sample preparation, demonstrated run times of 2 min per injection and can be fully automated. The method should therefore prove to be a valuable tool in the drug discovery process.

Keywords: LC/MS/MS; In vitro metabolism; Dextromethorphan; CYP 3A4

1. Introduction

Screening for drug metabolism is a very significant step in the process of developing a novel drug. It has several primary uses, to find metabolically stable compounds, define clearance pathways, predict in vivo human pharmacokinetics and drug–drug interactions [1,2]. Of particular relevance is the cytochrome (CYP) 3A family of enzymes and the polymorphically expressed CYP forms (e.g. CYP 2D6). Different techniques including fluorometric and radiometric have been used for in vitro metabolism screening [3–5] but these methods have potential limitations. A fluorometric assay, although superior to traditional assays, suffers from a lack of sensitivity for certain CYP forms [6]. Occasionally, interference by test compounds that are fluorescent or fluorescence modifying, may present problems [6]. Radiometric assays can be prohibitive in terms of cost of reagents, safety con-

* Corresponding author. Tel.: +1 804 828 3819; fax: +1 804 828 8359.
E-mail address: tom.karnes@vcu.edu (H.T. Karnes).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.08.043

cerns and inherent limitations that exist on miniaturization [7].

The availability of tandem mass spectrometers has provided drug metabolism scientists with a powerful tool for rapid analysis of biological samples for drugs and metabolites [8]. Rapid drug metabolite profiling can be achieved using fast chromatographic separation and mass spectrometric scanning without compromising separation efficiency. There have been a few investigations, which have used this approach to increase the throughput of various drug evaluation programs [6,9–12]. Most of these involve traditional in vitro incubation techniques and sample preparation steps that can significantly reduce the speed of analysis. Direct injection has been previously carried out for rapid bioanalytical determination of dextromethorphan in canine plasma [13] using LC/MS/MS but this approach involves no application for metabolism screening. Our goal was to develop a direct injection LC/MS/MS technique that involves no sample preparation and a novel incubation technique for the screening of the CYP 2D6 and the CYP 3A4 isozymes. LC separations were carried out on a short analytical column, 20 mm in length. The incubations were conducted in an autosampler tray equipped with a heating accessory. The microsomal incubation mixtures were directly injected onto the analytical column without any manipulation or addition of reagents to stop the incubation. Injections were made at an interval of every 2 min and the disappearance of the probe drug was monitored. Half-life ($t_{1/2}$) and intrinsic clearance ($Cl_{int\ in\ vitro}$) values were determined by measuring the first-order rate constant of elimination (K_e) at low concentrations of the probe drug ($\ll K_m$) [14,15]. The probe drugs chosen for our screening studies were dextromethorphan and midazolam, which are metabolized by CYP 2D6 and CYP 3A4 isoforms to form, dextrorphan and 1-hydroxy midazolam, respectively [16–18]. CYP 2D6 exhibits genetic polymorphism, which is a major source of inter-individual differences in drug metabolism [19]. CYP 3A4 is responsible for the biotransformation of numerous therapeutic agents and can therefore cause drug interactions [20]. Both these isozymes are therefore important for screening during the drug discovery process. The direct injection approach was tested for its screening potential by incubating dextromethorphan with known inhibitors of the CYP 2D6 isozyme including quinidine [21,22], chlorpromazine [23] and primaquine [24] in decreasing order of potency.

2. Experimental

2.1. Materials and reagents

Dextromethorphan (DM), midazolam maleate, quinidine hydrochloride, chlorpromazine hydrochloride, primaquine diphosphate were purchased from Sigma Chemicals (St. Louis, MO). Potassium phosphate (monobase and dibase) were both ACS grade chemicals and purchased from Sigma Chemicals (St. Louis, MO). Acetonitrile was of analytical grade and purchased from Baxter (Muskegon, MI). Pooled human (HLM) and rat liver (RLM) microsomes used in the incubation studies were purchased from Gentest Corporation (Woburn, MA). Deionized water used in the experiments was prepared in the laboratory using a Millipore Milli-Q reverse osmosis system (Bedford, MA).

2.2. Apparatus

The microsomal incubation mixtures were analyzed using a LC/MS/MS system that consisted of a Hewlett Packard Model 1100 series pump (Palo Alto, CA) coupled to a Finnigan LCQ mass spectrometer (San Jose, CA) fitted with either an atmospheric pressure chemical ionization source (APCI) or a electrospray ionization source (ESI). Xcalibur™ V 1.1 software was used for data acquisition and manipulation. Incubations were performed in a Perkin Elmer Model 200 autosampler equipped with a Peltier heating and cooling accessory. The column used for the LC analysis was a DASH C 18 column (2.0 mm i.d. × 20 mm length, 10 μm) purchased from Keystone Scientific (Bellefonte, PA).

2.3. Methods

2.3.1. LC/MS/MS method for the analysis of DM

A stock solution of dextromethorphan was prepared at a concentration of 10 mM in acetonitrile. Different dilutions were prepared from the stock in deionized water in concentrations ranging from 1 to 4 μM, which were used for method development. Dextromethorphan was analyzed in the positive ion mode using both the electrospray and the atmospheric pressure chemical ionization sources. In the ESI mode, LC separations were achieved on a DASH column (Section 2.2), and the mobile phase consisted of 0.1% formic acid in acetonitrile and water (30/70%) at a flow rate of 300 μl/min. The injection volume was optimized at 20 μl, and the run time was 2 min. MS/MS analysis was performed by isolating the protonated dextromethorphan at *m*/*z* 272, fragmenting it using a relative CE of 35% and scanning product ions from *m*/*z* 70 to 276. In the APCI mode, LC separations were performed on a similar DASH column. The mobile phase consisted of 0.1% formic acid of acetonitrile and water (22/78%) at a flow rate of 800 μl/min. The injection volume, analytical run time and MS/MS conditions were similar to those used in the ESI mode.

2.3.2. Feasibility of direct injection of the microsomal incubation mixtures

2.3.2.1. Qualitative matrix effects. This experiment was performed using a post-column infusion setup. Mobile phase was pumped through the column used for HPLC analysis and a syringe pump infused the analyte of interest (dextromethorphan 10 μM, rate 25 μl/min) into the post-column mobile phase stream via a mixing tee. This combined solution then entered the interface of the mass spectrometer. The mass spectrometer was operated in the product ion mode, wherein all fragments of the protonated drug were acquired. Subsequent

processing of the *m*/*z* 215 reconstructed ion chromatogram was used for data analysis.

A blank matrix consisting of 1 mg/ml HLM, 1 mM NADPH and 50 mM phosphate buffer (pH 7.4) was injected into the HPLC column. A neat standard injection of the analyte (dextromethorphan 1 μM) was then made onto the column without post-column infusion to establish the retention time. The post-column infusion experiment was performed with and without using the LCQ switching valve, which can direct the post-column LC eluent to waste or the interface of the mass spectrometer. When using the switching valve, the timing was varied between 0.25 and 0.50 min to establish the optimal time for the eluent to be switched back to the mass interface. The use of the switching valve reduces salt deposits on the ion source and increases the number of injections that can be made before contaminating the MS capillary interface.

2.3.2.2. Quantitative matrix effects. Matrix effects were determined by comparing the peak areas after injecting 1 μM dextromethorphan in water to the same concentration in microsomal incubation mixtures. Six injections of each sample were made onto the LC column. The sample injections were interwoven to account for any variability. A student's *t*-test was used to determine if there were any significant matrix effects at an alpha value of 0.05. The use of the column to desalt the incubation mixture was also established by comparing peak areas from an injection of 1 μM dextromethorphan in matrix onto the column versus response in the flow injection mode (no column). Three injections of each sample were made and the difference in peak areas was computed.

2.3.3. Performance of the column in the direct injection mode

Column performance in the direct injection mode was evaluated by using a new DASH column (10 μM, C18) and repeated injection of a sample of 1 μM dextromethorphan in matrix (1 mg/ml HLM and PB 50 mM, pH: 7.4). The first 100 injections used the switching valve at 0.25 min (optimized in Section 2.3.2.1). The next 100 injections did not use the switching valve. The change in the column backpressure, retention time and the peak areas of dextromethorphan were monitored over the entire period of 200 injections.

2.3.4. Direct injection analysis of dextromethorphan

2.3.4.1. Validation of precision and accuracy for the direct injection method. A standard curve was prepared by spiking different concentrations of dextromethorphan to a matrix containing 1 mg/ml HLM in 50 mM phosphate buffer. The concentrations of dextromethorphan used were 0.02, 0.05, 0.1, 0.5, 1.0, 2.0 and 4.0 μM. Four controls of the probe drug were also prepared in concentrations of 0.03, 0.08, 0.75 and 1.5 μM. The LC/MS/MS procedure remained the same as described in Section 2.3.1. The standards were injected in duplicate and the controls were injected six times each to determine the precision and accuracy of the method.

2.3.4.2. Comparison of incubation mixtures to controls. Incubation mixtures were prepared using 1, 5 or 10 μM dextromethorphan in 1 mg/ml human liver or rat liver microsomes, 1 mM NADPH and 50 mM phosphate buffer. The controls consisted of all the ingredients used in the incubation mixture except for the cofactor NADPH. The controls and the incubation mixtures were incubated in the autosampler at 37 °C and injections were made onto the LC column at 5 min intervals to monitor the change of dextromethorphan over time. The control corrects for losses such as adsorption or detector response; helping to ensure that metabolism is the only process that leads to a decrease in the peak area of the analyte.

2.3.4.3. Determination of $t_{1/2}$ for dextromethorphan in human and rat liver microsomes. Dextromethorphan was used in a concentration range of 0.1–2 μM for the determination of half-life in human liver microsomes. Incubation mixtures were prepared using 1 mg/ml HLM and 1 mM NADPH in phosphate buffer. These were incubated in the autosampler set at 37 °C. Direct injections were made from the sample vials at intervals of 2 min and the runs were monitored for a period of 35 min. The half-life was determined by plotting the peak areas of dextromethorphan on a logarithmic scale against time, which yielded the first-order rate constant for the disappearance of the probe drug. This was later converted to clearance values for dextromethorphan in human liver microsomes and compared values reported in the literature. A similar procedure was carried out to determine the half-life for dextromethorphan disappearance in rat liver microsomes using concentrations of the analyte in the range of 0.2–2.0 μM.

2.3.4.4. Four corner experiment. The objective of this experiment was to validate the use of the autosampler for conducting incubation studies. Vials containing the incubation mixture (1 mg/ml rat liver microsomes, 1 μM dextromethorphan, 1 mM NADPH in 50 mM phosphate buffer) were placed at the four corners of the autosampler (10 × 10 sample tray). The autosampler was used at temperature settings of 33, 37 and 41 °C. At each temperature setting, direct injections of the incubation mixture were made from the four corners at intervals of 2 min each. It was determined whether the four corners of the autosampler retained the same temperature by comparing the half-lives obtained from the four vials. This experiment also determined sensitivity to small changes in temperature by comparing the half-lives obtained at different temperatures.

2.3.4.5. Feasibility of high-throughput screening of the CYP 2D6 isozyme. A method should have the capability to screen for high, medium and low potency inhibitors of the enzyme system to be a successful screening approach. Quinidine,

chlorpromazine and primaquine were the three inhibitors of the CYP 2D6 isozyme used in decreasing order of their potency. Stock solutions (10 μM) of the three inhibitors were prepared using distilled deionized water and 1 μM of each was incubated separately with 2 μM of dextromethorphan, 1 mg/ml human liver microsomes, 1 mM NADPH in 50 mM phosphate buffer. Two control incubations were also run with the inhibition studies. Control I consisted of an incubation of dextromethorphan (2 μM) in matrix without NADPH and inhibitors and control II was an incubation of dextromethorphan (2 μM) without inhibitor. The five samples were placed in the autosampler, which was preset to 37 °C. The first two injections were made from the control incubations and successive injections were made from the other samples at intervals of 2 min each. The total run time for the experiment was 96 min and seven injections were made from each sample vial. The half-lives were calculated and compared to observe for inhibition. A strong inhibitor should significantly increase the half-life, whereas the half-life for the low potency inhibitor should be similar to that obtained in the incubation without inhibitors.

2.3.5. Direct injection analysis of midazolam

A 10 mM stock solution of midazolam was prepared in 50% acetonitrile and water. This solution was further diluted to 1 μM in deionized water to be used for method development. The LC method was developed in the isocratic mode using a (2.0 mm i.d. × 20 mm length, 10 μm, C18) DASH column. A mobile phase consisting of 0.1% formic acid in acetonitrile and water (30/70%) gave optimum separation. Detection was performed in the MS/MS mode. The protonated parent at *m*/*z* 326 was selected, fragmented at 35% relative collision energy and product ions scanned from *m*/*z* 82 to 330. The reconstructed ion chromatogram of the *m*/*z* 291 fragment was used for data analysis.

A negative control set of incubations was analyzed using 2 μM midazolam and 1 mg/ml HLM in 50 mM phosphate buffer. These negative controls lacked the cofactor NADPH. Injections were made from the control at intervals of 3 min to monitor any change in the peak area of the analyte. Incubations to determine the half-life were carried out using 2 μM midazolam, 1 mg/ml HLM and 1 mM NADPH in 50 mM phosphate buffer. Injections were made every 2 min and the decrease of the parent drug was monitored for a period of 10 injections. Incubations were carried out in the autosampler at 37 °C. Plots of the peak areas on a logarithmic scale versus time (min) were constructed to determine half lives and these were compared to literature values.

3. Results and discussion

3.1. LC/MS/MS method for the determination of dextromethorphan

The method for dextromethorphan was developed testing both ESI and APCI. The ESI method demonstrated an elution time of 0.8 min while the APCI had an elution time of 0.6 min. The ESI method was more sensitive than APCI (Fig. 1). The ESI source was therefore used for all our subsequent experiments and for determination of the precision and accuracy for dextromethorphan.

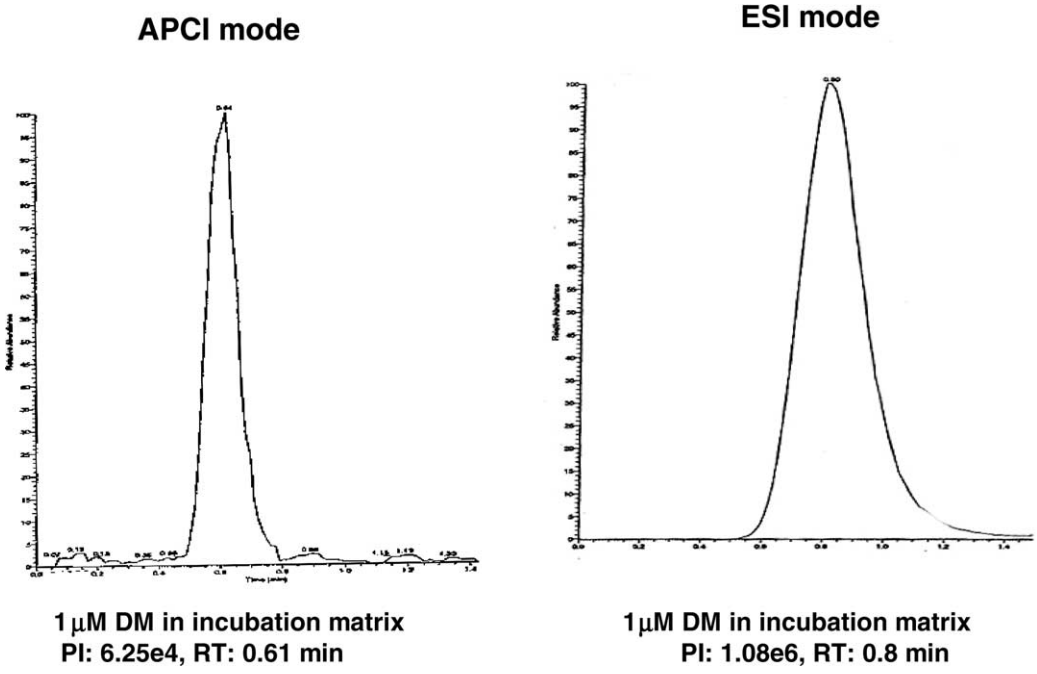

Fig. 1. Comparison of MS response from 1 μM dextromethorphan using ESI and APCI.

3.2. Feasibility of direct injection analysis of the microsomal incubation mixtures

3.2.1. Qualitative matrix effects

The baseline signal from the analyte infusion was altered when the blank matrix was injected onto the HPLC column. The effect was observed as a drop in the baseline based on the pre-injection response of the infused analyte. The extent of suppression and the length of time required to recover the pre-injection response of the analyte is shown in Fig. 2. The matrix alters the signal from the analyte between 0.20 and 0.65 min; the full signal for dextromethorphan is restored at around 0.7 min. This experiment was also performed using the switching valve, which directs the post-column eluent to the waste. Different time settings of the switching valve were used ranging from 0.25 to 0.5 min. It was discovered that a switching valve setting between 0.25 and 0.35 min restored the full signal of the analyte around 0.7 min. A time setting higher than 0.35 min depletes the analyte peak (retention time: 0.8 min), as it does not give the detector and its associated dead volume enough time to equilibrate before elution of the analyte.

3.2.2. Quantitative matrix effects

The effects on ESI response when injecting dextromethorphan (1 μM) in microsomal incubations versus deionized water provide a quantitative measure of the matrix effect. Without a switching valve, peak areas are similar (mean values: 2.04×10^7 dextromethorphan in deionized water and 1.84×10^7, dextromethorphan in incubation matrix). This was confirmed using the student *t*-test, which found the differences to be insignificant at a α value of 0.05. When using the switching valve at a time setting of 0.25, differences in peak area were even less (mean values: 2.24×10^7, dextromethorphan in deionized water and 2.16×10^7, dextromethorphan in incubation matrix) and found to be insignificant at an α

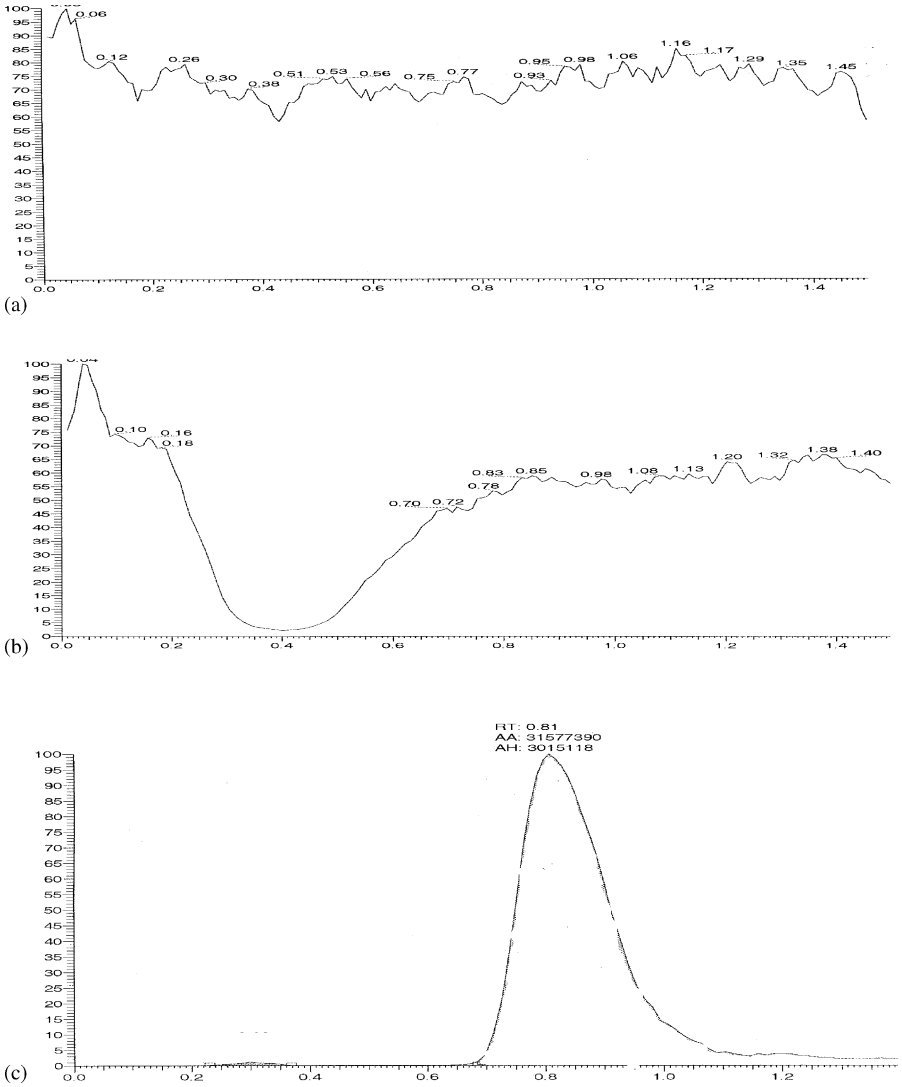

Fig. 2. Qualitative matrix effects obtained from the post-column infusion experiment. (a) Infusion of 10 μM dextromethorphan at 25 μl/min. (b) Direct injection of matrix (1 mg/ml HLM, 1 mM NADPH and 50 mM phosphate buffer). (c) Chromatogram of 2 μM dextromethorphan.

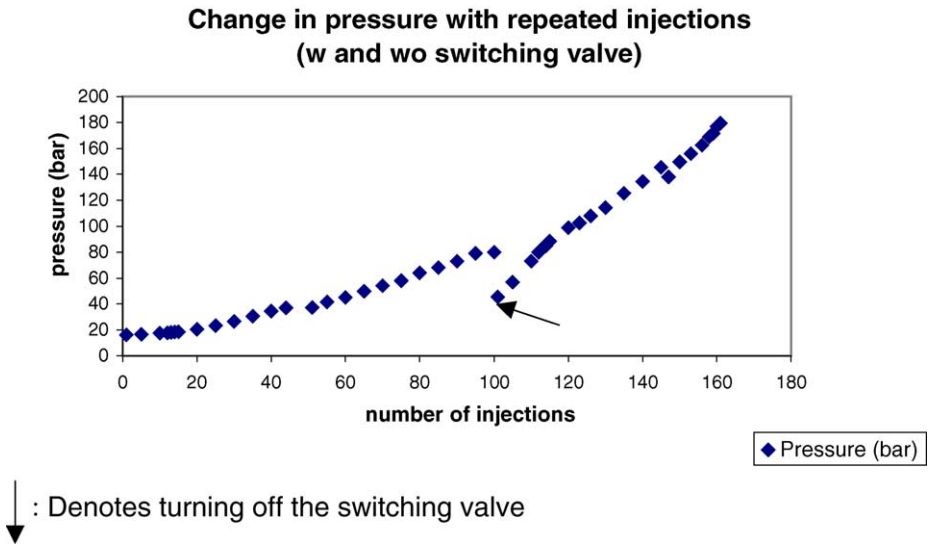

Fig. 3. Evaluation of column performance by monitoring changes in column backpressure.

value of 0.05. These results can be explained based on our qualitative matrix effects, which show that the analyte signal is restored around 0.70 min. A retention time of 0.80 min therefore, does not interfere with the direct injection analysis of dextromethorphan. The LC column can be effectively used for desalting the incubation mixture. The peak areas from injecting 1 μM dextromethorphan in microsomal incubation with and without using the LC column show an order of magnitude difference (mean values: 2.54×10^7, dextromethorphan with column and 1.84×10^6, dextromethorphan without column). Significant differences in the peak areas are observed, therefore necessitating the use of a column when performing direct injection analysis.

3.3. Performance of the column in the direct injection mode

The performance was evaluated by repeatedly injecting a sample of 1 μM dextromethorphan in matrix. The first 100 injections used the switch valve at a setting of 0.25 min and the next 100 injections were performed without use of this switch valve. A new column had a backpressure of 16 bar when the mobile phase (30/70%, acetonitrile/water) was pumped at a flow rate of 300 μl/min. The pressure rose successively after each direct injection made. Following 160 injections it reached 180 bar, which led to a breakdown in the chromatographic performance of the column. The peak area for dextromethorphan was also monitored, and this showed a slight decline after each direct injection. The changes in the backpressure of the column are shown in Fig. 3. During the course of the run, the retention time migrated from 0.80 to 0.69 min. This could be a possible reason for the decrease in peak area as the analyte peak steadily approaches the ion suppression zone. Another reason for decrease in peak area could be increased salt deposits on the ionization source and partial clogging of the MS interface on successive direct injections of the matrix. Therefore we have two end points to evaluate column performance, (1) the backpressure of the column and (2) the analyte retention time. Column backpressure should not exceed 180 bar and the analyte retention time should not migrate below 0.70 min to avoid matrix effects.

3.4. Direct injection analysis of dextromethorphan

3.4.1. Validation of precision and accuracy for the direct injection method

Different concentrations of dextromethorphan were prepared in the matrix and directly injected onto the column as described in Section 2.3.4.1 to form the standard curve. A linear model with a weighting factor of $(1/x^2)$ provided the best fit for calibration with a correlation coefficient of 0.999, and all residuals were less than 15%. The range of the assay was from 0.02 to 4.0 μM. The percent relative stan-

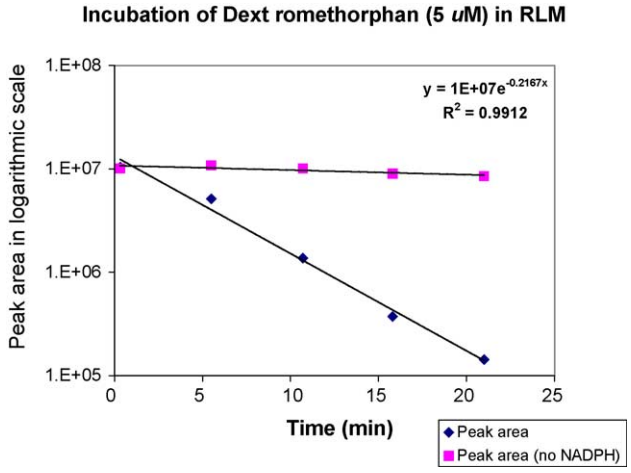

Fig. 4. Peak area in logarithmic scale vs. time plots of 5 μM dextromethorphan in RLM with and without (negative control) NADPH.

dard deviations (RSD) of the four controls, 0.03, 0.08, 0.75 and 1.5 μM were 6.69, 3.53, 6.10 and 3.73% and the percent differences from normal (DFNs) were −12.80, 2.95, −0.53 and −1.36%, respectively. The limit of quantitation was set at 0.02 μM, which represented the lowest point of the standard curve and this concentration had an RSD less than 15%.

3.4.2. Comparison of incubation mixtures to negative controls

Negative controls, which were prepared without the cofactor NADPH, demonstrated almost no change in the peak area of the analyte when monitored over a period of 20 min in RLM and 40 min in HLM. Incubation mixtures that contained NADPH showed a decline in the peak area of dextromethorphan due to its metabolism. Fig. 4 shows the comparison of control and incubation mixture for 5 μM dextromethorphan in RLM. This experiment demonstrates that metabolism is the only pathway for changes observed in the peak areas of the probe drug during incubation. It also facilitates data analysis, by simplifying the determination of $Cl_{int\,in\,vitro}$ for DM using the formula:

$$Cl_{int\,in\,vitro} = \frac{0.693}{t_{1/2}} \quad (\text{when } [S] \ll K_m)$$

The derivation of the formula is shown below.

Overall elimination or change in drug concentration over time is defined by

$$-\frac{dc}{dt} = K_e[S] \text{ and } K_e = \frac{0.693}{t_{1/2}} \quad (\text{first order elimination}) \tag{1}$$

In this case as the negative controls show no loss of drug and metabolism is the only path for elimination [28]

$$-\frac{dc}{dt} = \frac{V_{max}[S]}{K_m + [S]} \tag{2}$$

When $[S] \ll K_m$, the formula simplifies to [25]

$$-\frac{dc}{dt} = \left(\frac{V_{max}}{K_m}\right)[S] \quad \text{and} \quad Cl_{int\,in\,vitro} = \frac{V_{max}}{K_m} \tag{3}$$

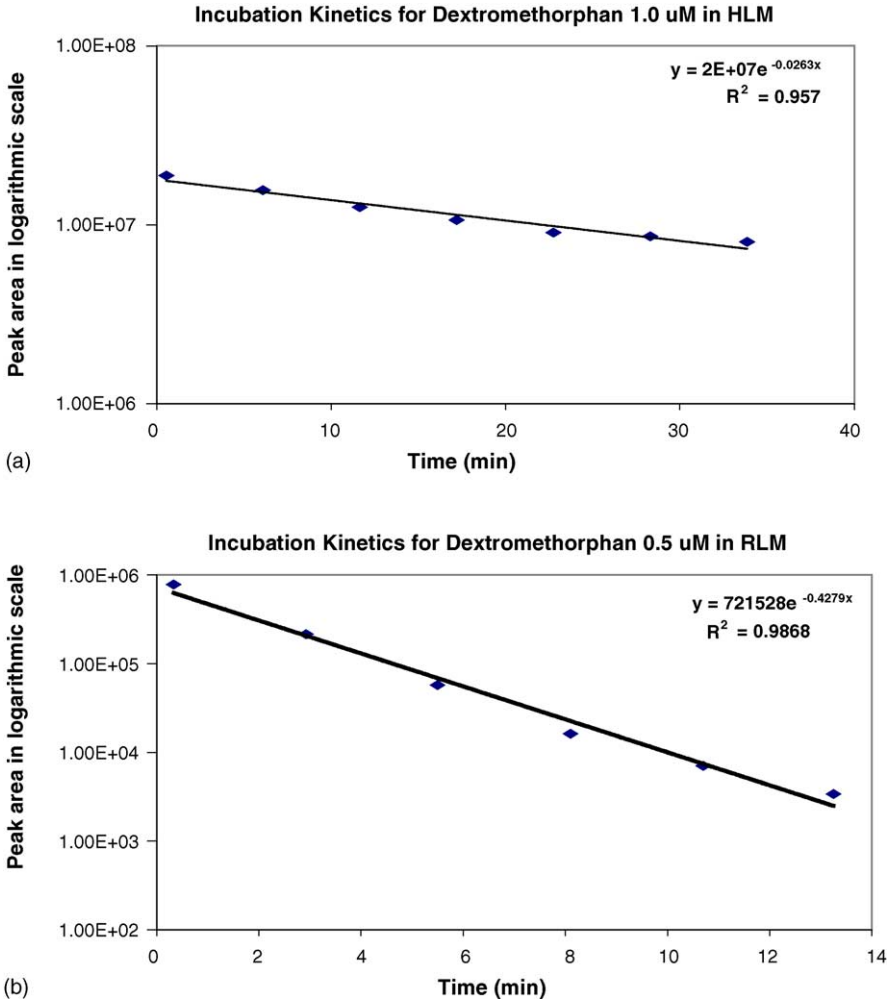

Fig. 5. (a) Kinetics of 1 μM dextromethorphan in HLM to determine its $t_{1/2}$. (b) Kinetics of 0.5 μM dextromethorphan in RLM to determine its $t_{1/2}$.

Table 1
Summary of half-lives and calculated intrinsic clearances of dextromethorphan in HLM[a]

Concentration DM (μM)[a]	Half-life (min)	$Cl_{int\ in\ vitro}$ (ml/(h mg))
0.1	20.5	2.03
0.2	25.1	1.66
0.8	27.3	1.52
1.0	26.4	1.58

DM: dextromethorphan; HLM: human liver microsomes.
[a] DM was used at concentrations of 0.1–1.0 μM. Concentrations greater than 1.0 μM were not used to determine $t_{1/2}$ as they were not significantly higher than the K_m values for DM in HLM, K_m 5.8–6.8 μM [26].

Comparing Eqs. (1) and (3) we see that

$$K_e = Cl_{int\ in\ vitro} = \frac{0.693}{t_{1/2}} \quad (4)$$

The K_e values are the slopes of the peak areas on a logarithmic scale versus incubation time plots. This is used to determine the $t_{1/2}$ (Eq. (1)), which is used in the estimation of the $Cl_{int\ in\ vitro}$ values (Eq. (4)) [25].

Table 2
Summary of half-lives and calculated intrinsic clearances of DM in RLM[a]

Concentration DM (μM)[a]	Half-life (min)	$Cl_{int\ in\ vitro}$ (ml/(h mg))
0.2	1.73	24.0
0.5	1.62	25.7

DM: dextromethorphan; RLM: rat liver microsomes.
[a] DM was used in concentrations of 0.2–2 μM. Concentrations greater than 0.5 μM were not used to determine $t_{1/2}$, as they were not significantly higher than values for DM in RLM, K_m: 1.3 and 1.76 [27,28].

3.4.3. Determination of $t_{1/2}$ for dextromethorphan in human and rat liver microsomes

Fig. 5 (top and bottom) illustrates representative plots (peak areas on a logarithmic scale versus incubation time) for the determination of the half-life for dextromethorphan in human and rat liver microsomes, respectively. Tables 1 and 2 summarize the results for the determination of the $t_{1/2}$ and $Cl_{int\ in\ vitro}$ in human and rat liver microsomes. The average $t_{1/2}$ and $Cl_{int\ in\ vitro}$ (mean ± S.D.) for the human liver microsomes was 25.46 ± 2.98 min and 1.65 ± 0.22 ml/(h mg) respectively. These values were comparable to those obtained in the literature for dextromethorphan in HLM using conventional incubation techniques

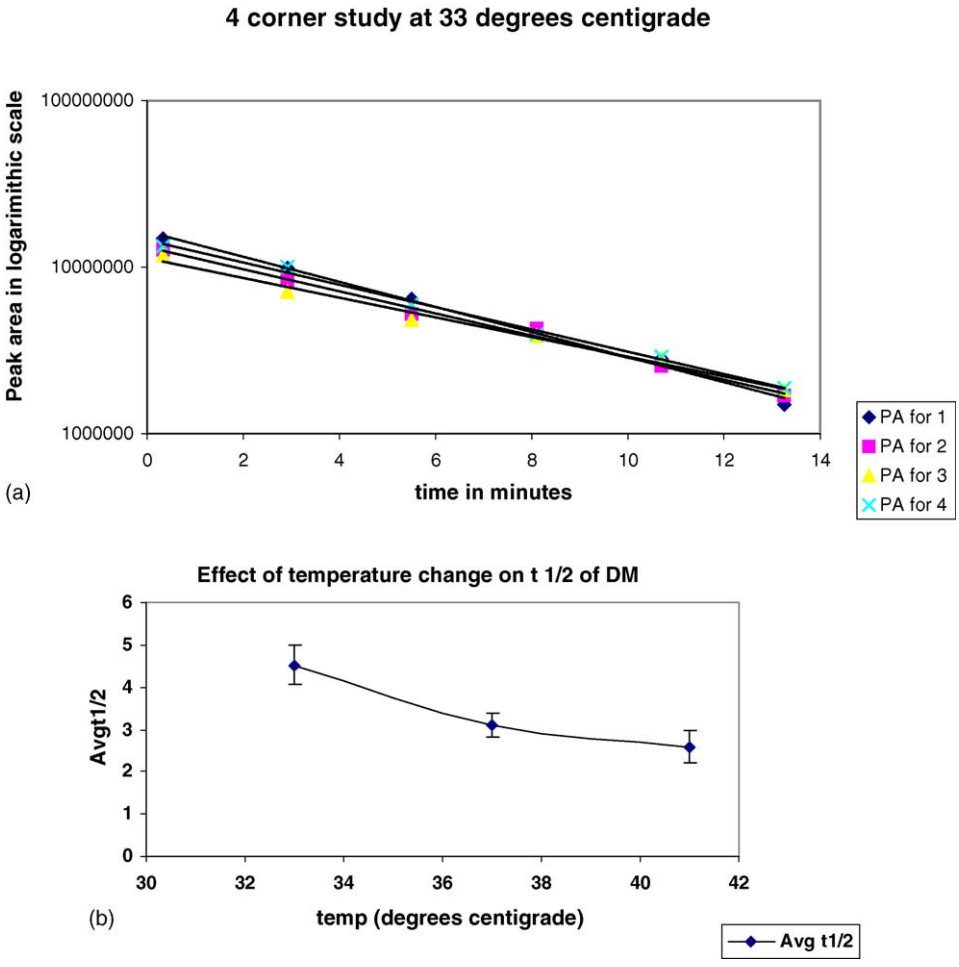

Fig. 6. (a) Incubation kinetics for the four corner study at 33 °C. (b) Changes in the $t_{1/2}$ values of dextromethorphan with increase in temperature.

($Cl_{int\ in\ vitro}$: 1.73–2.77 ml/(h mg) [26]). The average $t_{1/2}$ and Cl (mean ± S.D.) for the rat liver microsomes was 1.67 ± 0.05 min and 24.95 ± 0.69 ml/(h mg), respectively. The average $Cl_{int\ in\ vitro}$ values also compared favorably those previously reported in the literature ($Cl_{int\ in\ vitro}$: 20.04 and 25.38 ml/(h mg) [27,28]).

3.4.4. The four corner experiment

This experiment was performed in order to evaluate the capability of the Peltier heating accessory to maintain similar temperatures across the autosampler tray and also be sensitive to changes in temperature. Incubation studies were carried out from the four corners of the autosampler at three different temperatures (Section 2.3.4.4) to evaluate this. Fig. 6 (a) shows the representative plot at 33 °C for the half-lives of dextromethorphan obtained from the four corners of the autosampler. Fig. 6(b) shows the changes in half-lives by increasing the temperatures from 33 to 41 °C. The autosampler is a suitable alternative to conventional incubation techniques, which usually involve incubating in a water bath maintained at 37 °C upon continuous stirring. This is shown by the half-life experiment in which we obtained $t_{1/2}$ values comparable to those found in the literature for dextromethorphan in human and rat liver microsomes and by this experiment, which shows the ability of the autosampler to maintain uniform temperature throughout the sample tray.

3.4.5. Feasibility of high-throughput screening for the CYP 2D6 isozyme

Fig. 7(a) is a plot of the peak areas on a logarithmic scale versus incubation time for all the samples used in the screening studies with a total run time of 96 min. Table 3(a) summarizes the results obtained. The strong inhibitor of the CYP 2D6 isozyme (quinidine) increased the $t_{1/2}$ of dextromethorphan from 16.0 min (control II) to 20.2 min, an increase of 25.6%. The medium inhibitor chlorpromazine increased the $t_{1/2}$ by 6.14% and the weak inhibitor, primaquine had a $t_{1/2}$ that was not significantly different from the control incubation. Fig. 7(b) is a similar plot for the screening studies but this takes into consideration only the first four time points and hence a total run time of 48 min. Table 3(b) which summarizes these results show that the percentage increase in $t_{1/2}$ for the inhibitors is similar to those obtained with a run time of 96 min. Therefore, a run time of 48 min is sufficient to determine the potential for inhibition of the three inhibitors, which translates to a screening potential of 30 compounds

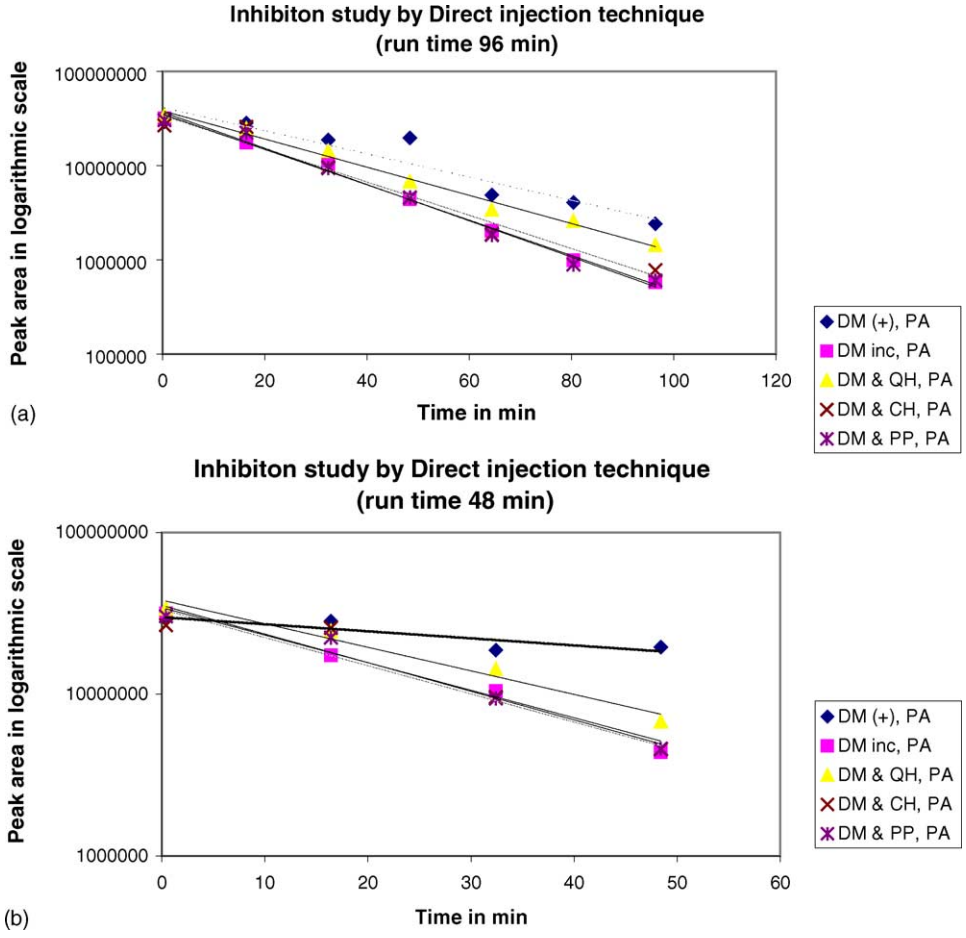

Fig. 7. (a) High-throughput screening for CYP 2D6 inhibition using three inhibitors and a total run time of 96 min. (b) High-throughput screening for CYP 2D6 inhibition using three inhibitors and a total run time of 48 min.

Table 3
Summary of inhibition results

Parameters	DM_{inc}	DM and QH	DM and CH	DM and PP
(a) Run time 96 min				
Half-lives	16.0	20.2	17.0	15.6
$Cl_{int\ in\ vitro}$ (ml/(min mg))	0.0432	0.0344	0.0407	0.0443
Increase in $t_{1/2}$ (%)	0.0	25.6	6.14	−2.48
(b) Run time 48 min				
Half-lives	17.3	20.7	17.6	17.0
$Cl_{int\ in\ vitro}$ (ml/(min mg))	0.0401	0.0335	0.0393	0.0407
Increase in $t_{1/2}$ (%)	0.0	19.7	2.05	−1.46

DM: dextromethorphan; QH: quinidine hydrochloride; CH: chlorpromazine hydrochloride; PP: primaquine diphosphate.

per working day. The number of compounds screened can be increased if we do not include the controls (i.e. compare the half lives obtained from the inhibitors to control half lives established previously) and reduce the inhibition experiments to three time points

3.5. Direct injection analysis of midazolam

The probe substrate for CYP 3A4 activity, midazolam was separated using 30/70% acetonitrile/deionized water with 0.1% formic acid at a flow rate of 300 μl/min. Under these conditions the, the retention time was approximately 0.87 min, which was removed from the matrix effects ($k' > 3$). Fig. 8 shows the comparison of negative control and incubation for midazolam (2.00 μM) in matrix (1 mg/ml HLM and 50 mM phosphate buffer) to determine its half-life. The negative control incubation (no NADPH) showed no decrease in the peak areas for midazolam over a time period of 25 min. This indicates that metabolism is the only pathway for the disappearance of the probe drug and the simplified formula (Section 3.4.2) can be used to determine intrinsic clearance.

The plot of the peak areas on a logarithmic scale versus incubation time was best described by a biexponential equation with an apparent first-order fast and slow (terminal) disposition rate constants. The $Cl_{int\ in\ vitro}$ was calculated from the initial rate constant [29] and the value obtained was 0.861 ml/(min mg). This value was comparable to previously reported values in the literature ($Cl_{int\ in\ vitro}$: 0.157–0.600 ml/(min mg) [30]). The concavity of the semi-log plot may be explained by assuming cross reactivity between the parent drug and its metabolites and thus later time points result in higher than expected concentrations for midazolam. Another reason for the increased concentration over later time points could be adsorption onto the column. A small

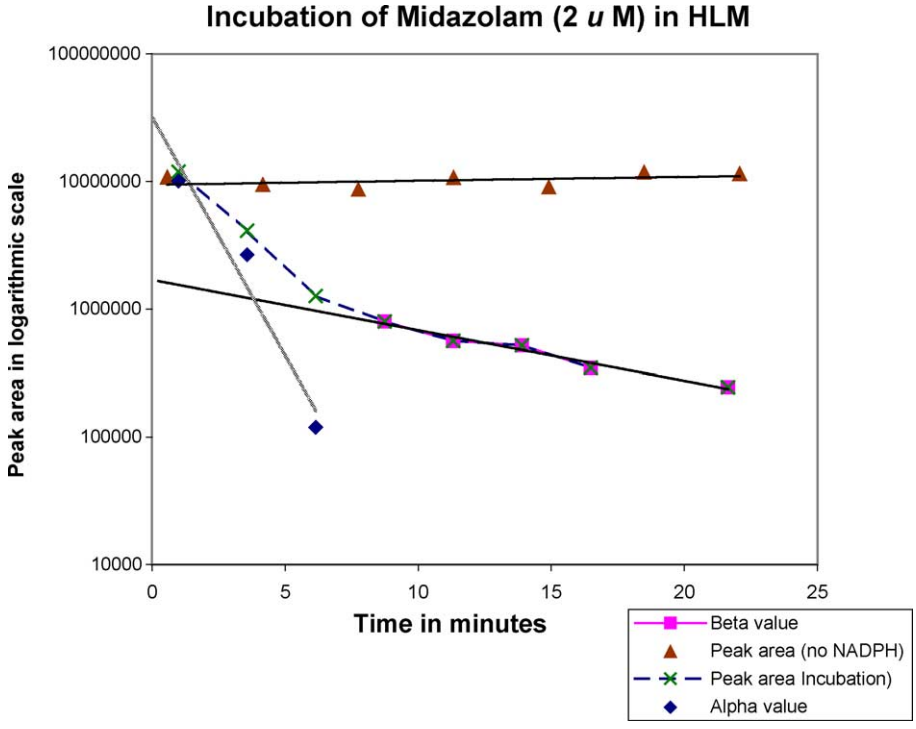

Fig. 8. Peak area in logarithmic scale vs. time plots of 2 μM midazolam in HLM with and without (negative control) NADPH.

increase in peak areas of the probe drug is also observed for the negative control (without NADPH) over increasing time points.

4. Conclusions

A LC/MS/MS method has been successfully developed for the determination of dextromethorphan and midazolam using a direct injection technique. The sensitivity achieved using ESI is greater than that obtained by using APCI for the direct injection approach. Provided sufficient attention is paid to matrix effects, fast and reliable analysis can be achieved. Direct injection and fast analysis times were made possible by using the DASH column, which had a relatively large particle size packing of C18 and a short length. Qualitative matrix effects showed that the matrix suppressed the analyte signal from 0.20 to 0.65 min but this was circumvented by eluting our peak at approximately 0.8 min ($k' > 3$). Column performance was evaluated and the lifetime of the column was set ca. 160 injections. Both the increase in backpressure and the migration of the retention time can be used as parameters to determine column lifetime.

Incubations were carried out in the autosampler, which was set to 37 °C using the Peltier heating accessory. The incubation technique differed from conventional techniques due to the absence of stirring and therefore experiments were conducted to validate this method. The $t_{1/2}$ for dextromethorphan and midazolam were calculated by incubating samples in the autosampler and the values determined showed agreement with those reported in the literature. Further validation of the autosampler was performed with the four-corner experiment, which showed that the autosampler maintains uniform temperature across the sample tray and that it is sensitive to changes in temperature. Screening was performed for inhibition of CYP 2D6 activity using established inhibitors in decreasing order of potency. The technique was able to differentiate between a strong and medium inhibitor of the isozyme and hence can be used for metabolism screening. Strong inhibitors are of most consequence in the screening process as they have potential for fatal drug interactions.

There is possibility of total automation using this technique as the incubations and the injections are conducted in the same autosampler and there is no need to stop the incubations after a certain amount of time as we determine half-life kinetics over different time points. Direct injection analysis involves no sample preparation and therefore increased the speed of analysis. The lack of sample preparation, short run times and the ease of use of this technique should make it an attractive tool in the early stages of drug discovery. This technique may also have value characterizing compounds that are rapidly metabolized.

References

[1] U.A. Meyer, J. Pharmacokinet. Biopharm. 24 (1996) 446–459.
[2] A.D. Rodrigues, Biochem. Pharmacol. 48 (1994) 2147–2156.
[3] C.L. Crespi, V.P. Miller, B.W. Penman, Anal. Biochem. 248 (1997) 188–190.
[4] N. Chauret, N. Tremblay, R.L. Lackman, J. Gauthier, J.M. Silva, J. Morris, J.A. Yergey, D.A. Nicoll-Griffith, Anal. Biochem. 276 (1999) 215–226.
[5] G.C. Moody, S.J. Griffin, A.N. Mather, D.F. McGinnity, R.J. Riley, Xenobiotica 29 (1999) 53–75.
[6] H. Yin, J. Racha, S.Y. Li, N. Olejnik, H. Satoh, D. Moore, Xenobiotica 30 (2000) 141–154.
[7] J.J. Burbaum, N.H. Sigal, Curr. Opin. Chem. Biol. 1 (1997) 72–78.
[8] E. Gelpi, J. Chromatogr. A 703 (1995) 59–80.
[9] R.B. Van Breemen, D. Nikolic, J.L. Bolton, Drug Metabol. Disposit. 26 (1998) 85–90.
[10] C.L. Fernandez-Metzler, K.G. Owens, T.A. Baillie, R.C. King, Drug Metabol. Disposit. 27 (1999) 32–40.
[11] J. Ayrton, R. Plumb, W.J. Leavens, D. Mallett, M. Dickins, G.J. Dear, Rapid Commun. Mass Spectr. 12 (1998) 217–224.
[12] H. Bu, L. Magis, K. Knuth, P. Teitelbaum, Rapid Commun. Mass Spectr. 14 (2000) 1619–1624.
[13] D.L. McCauley-Myers, T.H. Eichhold, R.E. Bailey, D.J. Dobrozsi, K.J. Best, J.W. Hayes II, S.H. Hohe II, J. Pharmaceut. Biomed. Anal. 23 (2000) 825–835.
[14] R.S. Obach, Drug Metabol. Disposit. 25 (1997) 1359–1369.
[15] R.S. Obach, Drug Metabol. Disposit. 27 (1999) 1350–1359.
[16] J. Ducharme, S. Abdullah, I.W. Wainer, J. Chromatogr. B 678 (1996) 113–128.
[17] T. Kronbach, D. Mathys, M. Umeno, F.J. Gonzalez, U.A. Meyer, Mol. Pharmacol. 36 (1989) 89–96.
[18] A. Ghosal, H. Satoh, E. Bush, P.E. Thomas, D. Moore, Drug Metabol. Disposit. 24 (1996) 940–947.
[19] U.A. Meyer, R.C. Skoda, U.M. Zanger, M. Heim, F. Broly, Pharmacogenetics of Drug Metabolism, Pergamon Press, New York, 1992, pp. 609–623.
[20] G. Wilkinson, J. Pharmacokinet. Biopharm. 24 (1996) 475–490.
[21] Y. Sai, R. Dai, T.J. yang, K.W. Krausz, F.J. Gonzalzez, H.V. Gelboin, M. Shous, Xenobiotica 30 (2000) 327–343.
[22] M.S. Ching, C.L. Blake, H. Ghabrial, S.W. Ellis, M.S. Lennard, G.T. Tucker, R.A. Smallwood, Biochem. Pharmacol. 50 (1995) 833–837.
[23] J. Shin, N. Soukhova, D.A. Flockhart, Drug Metabol. Disposit. 27 (1999) 1078–1084.
[24] C.M. Masimirembwa, J.A. Hasler, I. Johansson, Eur. J. Clin. Pharmacol. 48 (1995) 35–38.
[25] J.B. Houston, Biochem. Pharmacol. 47 (1994) 1469–1474.
[26] S.M. Abdel-Rehman, K. Marcuci, T. Boge, R. Russell, G.L. Kearns, J. Steven Leeder, Drug Metabol. Disposit. 27 (1999) 770–775.
[27] N.L. Kerry, A.A. Somogyi, G. Mikus, F. Bochner, Biochem. Pharmacol. 45 (1993) 833–839.
[28] L.E. Witherow, J.B. Houston, J. Pharmacol. Exp. Therapeut. 290 (1999) 58–65.
[29] R.S. Obach, A.E. Reed-Hagen, Drug Metabol. Disposit. 30 (2002) 831–837.
[30] M.F. Paine, M. Khalighi, J.M. Fisher, D.D. Shen, K.L. Kunze, C.L. Marsh, J.D. Perkins, K.E. Thummel, J. Pharmacol. Exp. Therapeut. 283 (1997) 1552–1562.