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Semi-automated liquid–liquid back-extraction in a 96-well format to decrease sample preparation time for the determination of dextromethorphan and dextrorphan in human plasma

Renee D. Bolden, Steven H. Hoke II, Thomas H. Eichhold, David L. McCauley-Myers, Kenneth R. Wehmeyer*

Procter and Gamble Pharmaceuticals, 8700 Mason-Montgomery Road, Mason, OH 45040, USA

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Abstract

A semi-automated, 96-well based liquid–liquid back-extraction (LLE) procedure was developed and used for sample preparation of dextromethorphan (DEX), an active ingredient in many over-the-counter cough formulations, and dextrorphan (DOR), an active metabolite of DEX, in human plasma. The plasma extracts were analyzed by liquid chromatography–tandem mass spectrometry (LC–MS–MS). The analytes were isolated from human plasma using an initial ether extraction, followed by a back extraction from the ether into a small volume of acidified water. The acidified water isolated from the back extraction was analyzed directly by LC–MS–MS, eliminating the need for a dry down step. A liquid handling system was utilized for all aspects of liquid transfers during the LLE procedure including the transfer of samples from individual tubes into a 96-well format, preparation of standards, addition of internal standard and the addition and transfer of the extraction solvents. The semi-automated, 96-well based LLE procedure reduced sample preparation time by a factor of four versus a comparable manually performed LLE procedure.

Keywords: Sample preparation; Dextromethorphan; Dextrorphan

1. Introduction

Sample preparation is often the rate limiting step in the development and application of methods for the trace analysis of drugs in biological matrices. The use of liquid chromatography–tandem mass spectrometry (LC–MS–MS) for bioanalytical methods often allows run times of a few minutes or less thereby increasing the demand for rapid sample preparation schemes. A recent trend to increase sample preparation throughput has been the use of parallel processing via the 96-well format. The development of liquid handling systems that are capable of processing samples in parallel using standard 96-well microtitre plate format has increased the speed, efficiency and accuracy of sample

*Corresponding author. Tel.: +1-513-622-2149; fax: +1-513-622-1196.

E-mail address: wehmeyer.kr@pg.com (K.R. Wehmeyer).

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preparation. A number of papers have been published on solid phase extraction sample preparation using parallel processing in the 96-well format [1–6]. A few 96-well liquid–liquid extraction (LLE) methods have also been reported but none of these methods have employed a back extraction [7–10]. Steinborner and co-workers [7,8] and Zhang et al. [10] have reported semi-automated 96-well LLE methods where both groups used a 96-channel liquid handling system for addition of the organic solvent. Jemal et al. [9] reported on the use of a liquid–liquid extraction method using methyl *tert*.-butyl ether as the extraction solvent and a four-channel liquid handling system. For all the reported methods, a dry down step was required since the methodology involved a direct extraction with no back extraction. Except for one method [9], a limiting factor was the need to manually transfer the samples from the original sample vials into the 96-well format.

Previously, we reported a manual LLE back-extraction procedure for the isolation of dextromethorphan (DEX) and dextrorphan (DOR) from human plasma prior to analysis by a stable-isotope dilution-based LC–MS–MS method [11]. Currently, we are reporting the development of a semi-automated LLE back-extraction procedure based on a parallel 96-well format for the preparation of DEX and DOR in human plasma samples prior to LC–MS–MS analysis. A 12-channel Hamilton Microlab AT Plus 2 liquid handling system was utilized to perform all liquid transfer steps in the extraction method. The methodology involves an ether extraction of DEX and DOR from plasma, followed by a back extraction from the ether layer into acidified water. The Microlab has several key features that make it an ideal instrument to perform parallel LLE methods. First, the Microlab uses plunger-in-tip positive displacement pipette tips that permit the accurate and precise transfer of standards, internal standards, samples and volatile organic solvents without dripping. The plunger-in-tip design allows the pipetting of high-volatility organic solvents without dripping and the design has no seals which can be eroded over time by the organic solvents typically used for LLE. Additionally, the 12-channel design allows for rapid parallel liquid manipulations. Method performance including accuracy, precision, absolute recovery, suppression, cross-contamination, stability and ruggedness were examined. The methodology was also applied to the analysis human pharmacokinetic samples.

2. Experimental

2.1. Chemicals and reagents

DEX (see Fig. 1A), was obtained from the United States Pharmacopeial Convention (Rockville, MD, USA) and DOR (see Fig. 1C) was obtained from Research Biochemicals International (Natick, MA, USA). The stable isotope-internal standards, [$^{2}H_{3}$]DEX (SIL-DEX: see Fig. 1B) and [$^{2}H_{3}$,^{13}C]DOR (SIL-DOR: see Fig. 1D), were prepared at Procter and Gamble Pharmaceuticals Health Care Facility (Mason, OH, USA). [*N*-Methyl-^{3}H]dextromethorphan (^{3}H-DEX) was obtained New England Nuclear (Boston, MA, USA). Ethyl ether (reagent grade), sodium hydrogencarbonate (reagent grade) and formic acid (SupraPur) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Blank human plasma was obtained from volunteer donors at Procter and Gamble. Ultima Gold scintillation cocktail was purchased from Packard Instrument (Meridan, CT, USA) and distilled–deionized water was obtained from a Barnstead NanoPure II system (Dubuque, IA, USA).

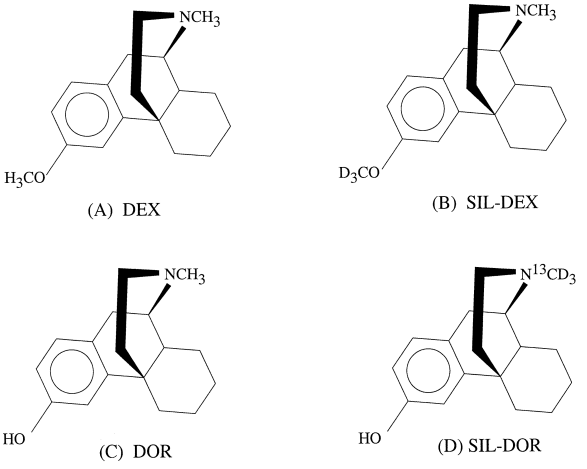

Fig. 1. Structures of (A) dextromethorphan (DEX), (B) [$^{2}H_{3}$-*O*-methoxy]dextromethorphan (SIL-DEX), (C) dextrorphan (DOR) and (D) [$^{2}H_{3}$,^{13}C-*N*-methyl]dextrorphan (SIL-DOR).

2.2. Instrumentation for sample handling and LLE

A MicroLab AT Plus 2 (Hamilton, Reno, NV, USA) was used to perform all liquid transfers during the LLE back-extraction method. The MicroLab was used for manipulating samples from a single tube format into the 96-well plate format, preparing standards and for addition of internal standard to the samples, quality controls (QCs) and standards. Furthermore, the Microlab was used for the addition of organic solvent and buffer used for the extraction procedures, as well as for the transferring of ether and acid solutions from the extraction steps to clean polypropylene tubes. To achieve the best accuracy and precision, the standard and internal standard solutions were dispensed 0.3 mm from the bottom of the polypropylene tubes. The standards, internal standard and buffer were aspirated using liquid-level sensing with a single pre-wet mixing step before solution transfer. The transferring of ether was performed using a pre-set depth without liquid-level detection.

2.3. Preparation of DEX and DOR plasma standards

Initial stock solutions of DEX, DOR, SIL-DEX, and SIL-DOR were prepared in methanol. Combined DEX/DOR stock standard solutions and combined SIL-DEX/SIL-DOR stock internal standard solutions were prepared in 0.1% sodium chloride–methanol (50:50, v/v) and stored at −20 °C. Sodium chloride was added in order to use liquid-level sensing on the MicroLab instrument. Working plasma standards were prepared daily by first pipetting 20 μl of a 25 ng/ml SIL-DEX/SIL-DOR combined stock solution (500 pg) into a rack of 96 empty polypropylene tubes (1.1 ml, National Scientific Supply, Claremont, CA, USA), followed by 20 μl of a 1 *M* sodium carbonate buffer (pH 10.54). Then, 10 μl of the appropriate DEX/DOR combined standard stock was added to selected tubes to provide DEX and DOR masses ranging from 1.64 to 4100 pg/tube and 1.36 to 3410 pg/tube, respectively. Finally, an aliquot (0.2 ml) of blank human plasma was added to each standard tube providing DEX standards covering a concentration range from 8.2 to 20 500 pg/ml and DOR standards covering a concentration range from 6.8 to 17 050 pg/ml. Replicate standards ($n=2$) were prepared for the two lowest and two highest standards while the remaining standards were prepared singly on each analysis day.

2.4. Preparation of quality control samples

A combined DEX/DOR QC stock sample (DEX= 13 120/DOR=10 920 pg/ml) was prepared by adding a small aliquot of the appropriate DEX/DOR combined stock standard solution to a 10-ml volumetric flask and diluting to volume with blank human plasma. Subsequent combined QC stock samples were prepared by serial dilution with blank human plasma to give DEX/DOR levels of 1312/1092 and 131.2/109.2 pg/ml DEX/DOR. The combined stock QC plasma samples were stored at −80 °C. On each validation day, working QC samples were prepared in a 96-well plate format using the MicroLab. An aliquot (0.2 ml) of each QC stock solution was pipetted into a series of polypropylene tubes already containing 20 μl of the combined SIL-DEX/SIL-DOR solution (25 ng/ml) and 20 μl of the 1 *M* sodium carbonate, pH 10.54 buffer. On each validation day, replicate ($n=24$) working QC samples were prepared at each level.

2.5. Preparation of study samples

Plasma samples obtained from subjects dosed with Vicks Formula 44 cough syrup (containing 30 mg of DEX/HBr), were prepared for analysis in a 96-well plate format using the MicroLab instrument. An aliquot (0.2 ml) of individual subject samples was added into separate tubes already containing 20 μl of a 25 ng/ml SIL-DEX/SIL-DOR combined solution and 20 μl of the 1 *M* sodium carbonate, pH 10.54 buffer. For study samples expected to contain concentrations of DEX/DOR higher than the range of the standard curve, a smaller volume of the sample was aliquoted into the polypropylene tube and volumetrically diluted with blank human plasma.

2.6. LLE sample preparation

A 600-μl aliquot of ethyl ether was added to each well of a 96-well rack of plastic tubes containing the standards, QCs and study samples using the Mi-

crolab liquid handling system. The tubes contained in the 96-well rack were then covered with a 96-well mat cap tube sealer (Microliter, Suwanee, GA, USA). An aluminum block was placed on top of the mat cap and the entire assembly was placed in a multi-tube vortex (VWR, So. Plainfield, NJ, USA) and clamped into place. DEX and DOR were subsequently extracted into the ether layer by vortexing using the multi-tube vortex at mid-level power for 5 min.

Following extraction, the tubes in the 96-well rack were placed in a dry ice–acetone bath to freeze the plasma layer. Freezing the aqueous layer simplified the transfer of the ether without the possibility of transferring plasma. Additionally, freezing the aqueous layer minimized the potential for cross-contamination between the sample tubes by condensing the ether vapors away from the top of the closely spaced sample tubes. It was important to keep the mat cap cover secured with the aluminum block during the freezing step in order to prevent the ether pressure from pushing the mat cap out of the tubes.

After the plasma layer was frozen, the mat cap was carefully removed and a portion of the ether layer (400 μl) was transferred (using the Microlab) to a 96-well rack of clean polypropylene tubes that already contained 200 μl of 1% formic acid. The unique plunger-in-tip design of the Hamilton Microlab instrument prevents problems of ether dripping from the tips as is often encountered with air displacement pipet systems. The samples tubes were again covered with the mat cap/aluminum block configuration and back-extracted for 5 min into the acidified water using the multi-tube vortexer. Finally, the Microlab was used to transfer a portion (150 μl) of the acidified extract to clean autosampler vials. The plunger-in-tip design pipette tips of the Microlab permit the transfer of the bottom acidified water layer without the possibility of ether drawing into the tips. Following the sample preparation step, the standards, QCs and samples were analyzed by LC–MS–MS as described below.

2.7. Absolute recovery of DEX and DOR from plasma using LLE

The absolute recovery of DEX and DOR from the LLE procedure was evaluated using blank human plasma spiked with DEX at the 131.2 and 13 120 pg/ml levels and with DOR spiked at the 109.2 and 10 920 pg/ml levels. Replicate ($n=5$) aliquots, without internal standard, were carried through the LLE sample preparation procedure and 150 μl of the acidified extract was added to autosampler vials already containing 20 μl of the 25 ng/ml SIL-DEX/SIL-DOR combined internal standard solution. The samples were then analyzed by LC–MS–MS and the concentration of DEX and DOR were determined from the linear regression curve. The absolute recoveries of DEX and DOR were calculated by dividing the concentration of DEX and DOR found from the analysis by the expected concentration of DEX and DOR and multiplying by 100. Five different plasma sources were evaluated at each concentration.

2.8. Stability of DEX/DOR in sample extracts

A set of standards and QCs ($n=24$ for all three QC levels) were prepared using the described LLE procedure. The samples and the standards were analyzed on the day of preparation and subsequently analyzed a second time after storage at 4 °C for 1 week. The stability of the prepared samples was determined by comparing the results obtained after 1 week with those obtained on the initial day of analysis.

2.9. Signal suppression of DEX/DOR during analysis of sample extracts

The potential suppression of the electrospray signal by components in the prepared plasma sample matrix was determined through comparison of spiked blank plasma extracts with spiked acidified water solutions. Aliquots (0.2 ml) of blank plasma from eight subjects (four female and four male) were extracted using the described LLE procedure. The acidified extracts (150 μl) were transferred to autosampler vials already containing 20 μl of a 25 ng/ml SIL-DEX/SIL-DOR internal standard solution and 10 μl of a 4.1/3.4 ng/ml DEX/DOR combined standard solution. The neat acidified water solutions were prepared by adding 20 μl of a 25 ng/ml SIL-DEX/SIL-DOR internal standard solution and 10 μl of a 4.1/3.4 ng/ml DEX/DOR combined

standard solution to 150 μl of 1% formic acid. Samples were prepared in replicate ($n=4$) for each subject. Suppression caused by the plasma matrix was determined by dividing the peak area for the analyte from the spiked plasma extracts by the peak area of the corresponding analyte obtained from neat acidified aqueous spike solutions.

2.10. Cross-contamination

Cross-contamination between samples is always a concern when using a 96-well format due to the close proximity of the sample tubes. To evaluate potential cross-contamination, ^{3}H-DEX (150 000 dpm/200 μl) spiked plasma samples were alternately placed in the wells between blank plasma samples creating a checkerboard pattern on a 96-well rack of tubes. This configuration was used to yield the maximum potential of cross-contamination by surrounding blank samples with the samples spiked with high levels of ^{3}H-DEX. The samples were then processed using the LLE procedure as described. After the entire LLE procedure, the acidified water extract of the blank samples was transferred to a scintillation vial, mixed with Ultima Gold scintillation cocktail and counted using a Packard Liquid Scintillation Analyzer 2550TR/LL. The high level of ^{3}H-DEX used in the spiked plasma solutions would allow the detection of a 0.025% cross-contamination in the blank samples.

2.11. Accuracy and precision

QC samples were analyzed on three separate days to determine the accuracy and precision of the overall methodology. For each validation day, replicate ($n=24$) working QC samples were prepared at each QC level (DEX/DOR levels: 131.2/109.2, 1312/1092 and 13 120/10 920 pg/ml) using the LLE procedure previously described.

2.12. LC–MS–MS conditions

A Gilson (Middletown, WI, USA) modular HPLC system consisting of a Model 308 control pump, two Model 306 auxiliary pumps, a Model 811C dynamic mixer, a Model 821 pressure regulator, and a Model 234 autoinjector comprised the chromatographic system. The analytes were retained and eluted on a Waters, 50×2.1 mm, 3.5 μm Symmetry C_8 column. However, two separate HPLC methods were used for the analysis of DEX and DOR. For DEX, the mobile phase was water–methanol–formic acid (52:48:0.1, v/v/v) and for DOR, the mobile phase was water–methanol–formic acid (68:32:0.1, v/v/v). The flow-rate and injection volume for each method were 350 μl/min and 20 μl, respectively. The entire chromatographic eluent was passed into the mass spectrometer interface for subsequent detection. Under these conditions, the HPLC retention times for DEX and DOR were ~0.5–0.7 min.

The mass spectrometer was a Perkin-Elmer Sciex API III^{+} (Thornhill, Canada) operated in the TurboIonSpray configuration, consisting of the articulated IonSpray inlet used in conjunction with the heated TurboProbe desolvation unit. The TurboProbe temperature and nitrogen gas flow rate were 480 °C and 8 l/min, respectively. The nebulizer gas pressure was 60 p.s.i. (nitrogen) (1 p.s.i.=6894.76 Pa). Protonated analyte ions were generated using electrospray ionization (ESI) with orifice potentials of 4000 and 80 V, respectively. Collisional activated dissociation (CAD) was achieved using argon as the collision gas, at a thickness of $300 \cdot 10^{13}$ molecules/cm^2 and a collision energy of 30 and 27 eV for DEX and DOR, respectively. The selected reaction monitoring (SRM) transitions m/z 272→147 and m/z 275→150 were sequentially monitored for detection of DEX and SIL-DEX, respectively, while SRM transitions m/z 258→201 and m/z 262→201 were sequentially monitored for detection of DOR and SIL-DOR, respectively. The dwell time for each transition was 215 ms, yielding a scan rate of two scans/second. Peak areas for the chromatographic peaks were determined using the PE-Sciex software package MacQuan, Version 1.5.

2.13. Quantitation of DEX and DOR

Calibration curves were constructed by plotting the peak area ratios (DEX/SIL-DEX) for standards versus DEX concentrations and fitted using a weighted ($1/x$) regression line within the MacQuan software package. Drug concentrations in the QC and unknown samples were then interpolated from the

weighted linear regression curve. Calibration curves for DOR were prepared analogously.

2.14. Ruggedness

In a normal assay batch up to three 96-well plates of samples are prepared and analyzed. Thus, the ruggedness of the methodology was evaluated for the large number of samples to be analyzed. Ruggedness of the column was determined by analyzing standards and quality controls samples on a single column. A new column was employed when peak shape degraded or when the methodology did not produce acceptable results.

2.15. Human pharmacokinetic study

Volunteers were dosed with commercially purchased Vicks Formula 44 cough syrup (Procter and Gamble, Cincinnati, OH, USA) containing 30 mg of DEX/HBr. Blood samples (10 ml) were subsequently obtained at 0, 0.16, 0.33, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 16 and 26 h post oral dose. The blood was immediately placed on ice and subsequently processed by centrifugation to yield plasma. The resulting plasma samples were then stored in 2-ml polypropylene cryovials at −80 °C until analysis.

3. Results

3.1. LC–MS–MS spectra

The ESI mass spectra obtained for both analytes and their corresponding internal standards have been reported previously [11] and will be briefly reviewed. DEX, SIL-DEX, DOR and SIL-DOR spectra were dominated by intense protonated molecular ions at m/z 272, 275, 258 and 262, respectively. The SRM transitions schemes chosen for each compound were m/z 272→147 for DEX, m/z 275→150 for SIL-DEX, m/z 258→201 for DOR and m/z 262→201 for SIL-DOR (Fig. 2). Previously [11], DOR was monitored using m/z 258→199 but the 258→201 transition provides an incremental improvement in selectivity with only a slight loss in sensitivity.

3.2. Chromatographic profiles of blank and DEX/DOR spiked human plasma

The mobile phase conditions were optimized for each analyte so that DEX and DOR would elute between 0.5 and 0.7 min. The LC–MS–MS SRM profiles generated under typical conditions are shown in Fig. 3 for blank plasma extracts and for samples containing 8.2 pg/ml DEX and 6.2 pg/ml DOR. With the instrumentation employed in this study, an optimal combination of sensitivity and sample throughput was achieved using two separate isocratic methods rather than a single isocratic or gradient method. Under the rapid analysis conditions, the blank plasma extract contained no interferences and enabled the detection of trace levels of DEX or DOR. Similarly, chromatograms of blank plasma for SIL-DEX and SIL-DOR were free from interferences (data not shown).

3.3. Calibration curves

Calibration curves for DEX and DOR were fitted using a weighted $(1/x)$ regression over three orders of magnitude, with correlation coefficients for the regressions being 0.996 or greater. Typically, the back-calculated concentrations for the standards were 100±15% of expected across the entire standard range.

3.4. Speed of 96-well LLE procedure

The semi-automated parallel LLE back-extraction using a 96-well format greatly reduced the time required for sample preparation. The previous manual method [11] required ~8 h for the preparation of 100 samples while the current semi-automated 96-well parallel approach is capable of preparing 96 samples in 2 h including steps such as uncapping vials, arraying vials on the instrument and capping sample vials after removing aliquots of plasma. The use of the MicroLab pipetting system and the 96-well format avoids the labeling of multiple tubes, allows pipetting of 12 samples or reagents simultaneously and easily permits the parallel extraction of 96 samples. A limitation of the current 96-well approach is sample size and extraction volumes are

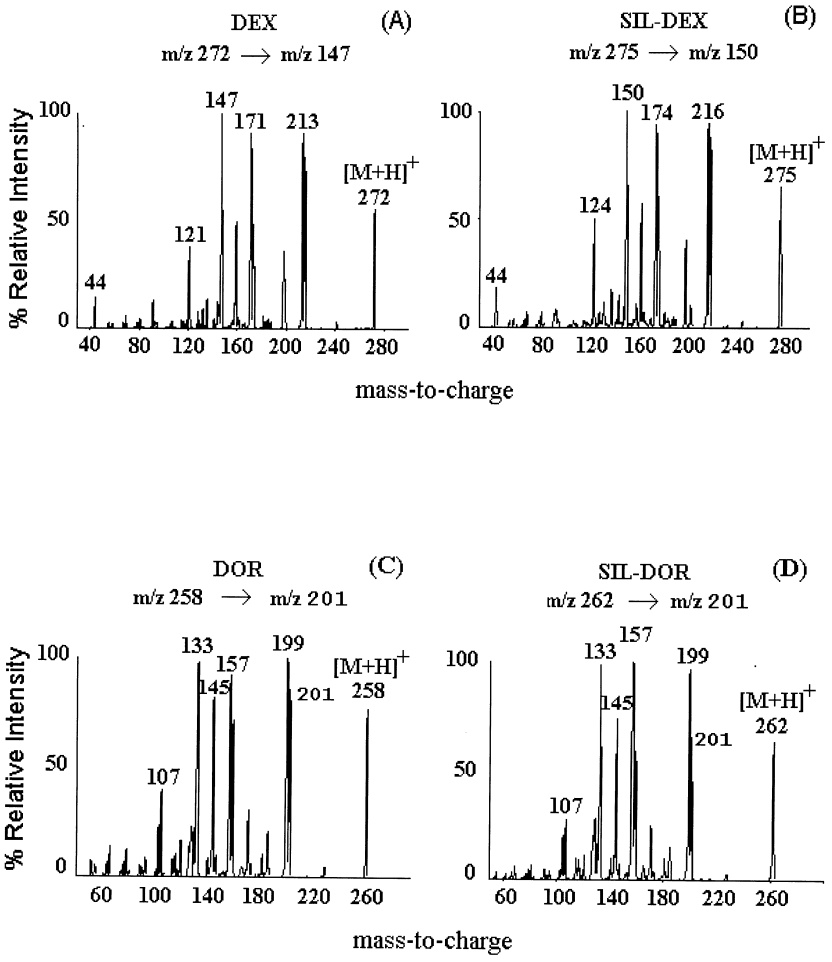

Fig. 2. Electrospray product ion spectra of (A) DEX, (B) SIL-DEX, (C) DOR and (D) SIL-DOR.

limited due to a maximum volume of 1.1 ml for commercially available 96-rack tubes.

3.5. Absolute recovery of DEX and DOR by LLE

Although the use of the stable isotope-labeled internal standards, SIL-DEX and SIL-DOR, would correct for any loss of DEX or DOR during the sample preparation procedure, it was still of interest to determine the recovery of the drug during sample preparation in order to aid in assessing the robustness of the methodology. The absolute recovery of DEX and DOR during sample preparation was examined by processing plasma samples ($n=5$) that were spiked with only DEX and DOR, at two separate levels (DEX: 131.2 and 13 120 pg/ml; DOR: 109.2 and 10 920 pg/ml), through the LLE back-extraction step. The formic acid back extract solution was then spiked with the mixed internal standard solution to determine the absolute recovery of DEX and DOR. Plasma from five separate volunteers was examined in this manner. The absolute recovery of DEX extracted into the formic acid was determined to vary between 25.2 and 45.7% with an RSD of 3.6–16.2% for the 131.2 pg/ml spike and 39.3–57.1% with an RSD of 2.4–8.6% for the 13 120 pg/ml spike (Table 1). The absolute recovery of DOR following the LLE back-extraction step was determined vary between 29.1 and 62.5% with an RSD of 6.1–13% for the 109.2 pg/ml spike and 45.2–53.7% with an RSD of

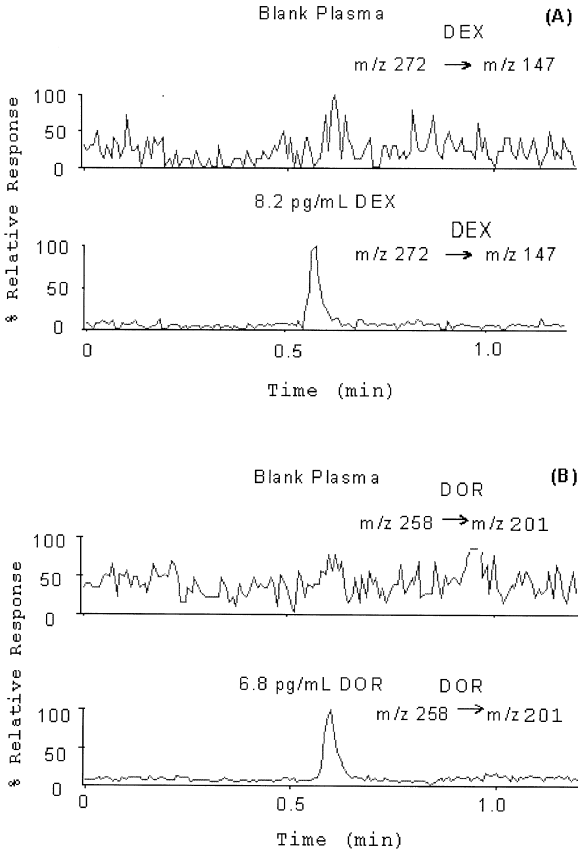

Fig. 3. LC–MS–MS profiles for blank human plasma extracts and spiked human plasma extracts (A) 8.2 pg/ml DEX and (B) 6.8 pg/ml DOR.

Table 1
Absolute recovery of dextromethorphan from human plasma (n=5)

Spiked [DEX] (pg/ml)	Volunteer	Average recovery (RSD) (%)
131.2	A	25.2 (16.2)
131.2	B	44.5 (7.0)
131.2	C	41.2 (3.6)
131.2	D	32.2 (10.1)
131.2	E	45.7 (9.2)
13 120	A	39.3 (2.4)
13 120	B	52.7 (5.0)
13 120	C	45.4 (6.3)
13 120	D	43.2 (8.6)
13 120	E	57.1 (5.6)

Table 2
Absolute recovery of dextrorphan from human plasma (n=5)

Spiked [DOR] (pg/ml)	Subject	Average recovery (RSD) (%)
109.2	A	29.1 (8.4)
109.2	B	62.5 (8.8)
109.2	C	50.2 (6.1)
109.2	D	44.9 (10.0)
109.2	E	60.1 (13.0)
10 920	A	48.2 (7.5)
10 920	B	53.7 (9.4)
10 920	C	47.9 (6.0)
10 920	D	45.2 (4.1)
10 920	E	52.7 (6.3)

4.1–9.4% for the 10 920 pg/ml spike (Table 2). The absolute recovery of DEX and DOR was found to show some subject-to-subject variability, however, within a given subject the recovery was fairly consistent. The reported recoveries do not correct for the fact that only 2/3 of the ether and 3/4 of the formic acid were removed for analysis. Volume correction to calculate the efficiency of extracting DEX and DOR into the ether and formic acid layers would yield recovery values closer to 100%.

3.6. Stability of extracted DEX and DOR samples

The stability of DEX and DOR in the 1% formic acid back extraction solvent was confirmed by analyzing extracts from QC plasma samples spiked with 131.2, 1312, and 13 120 pg/ml DEX and 109.2, 1092 and 10 920 pg/ml DOR. Both the QC samples (n=24 at each level) and their corresponding standards were analyzed initially and after 1 week of storage at 4 °C. The concentration of DEX and DOR in the QC samples were determined from the original standard curve that had been stored with the QC samples. After 7 days of storage, the DEX QC samples were within 5% of their initial measured values for the DEX while the DOR samples were within 8% of their initial measured values. The results demonstrate both DEX and DOR are stable in sample extracts.

3.7. Matrix suppression

Matrix suppression of the electrospray ionization of DEX and DOR was determined for both male and

female human plasma extracts. The peak areas obtained for both DEX and DOR spiked into the 1% formic acid extract obtained from blank plasma were compared to the peak areas obtained from DEX and DOR spiked at the same level into 1% formic acid. The average percent suppression was calculated for the four male and four female plasma samples. The male plasma samples exhibited 21.9 and 23.4% average signal suppression for DEX and DOR, respectively. The female plasma samples exhibited a slightly increased average signal suppression of 29.1 and 36.3% for DEX and DOR, respectively. Even with the rapid elution times, the LLE back-extraction procedure resulted in extracts that exhibited minimal suppression during the ionization process.

3.8. Cross-contamination

The potential for cross-contamination between samples is a concern in 96-well based techniques due to the close spatial proximity of the sample tubes. Two factors were found to be important in order to minimize cross-contamination in the LLE back-extraction procedure. First, the mat cap used to seal the samples during mixing must be secured with the aluminum block during the mixing and freezing steps. The ether vapors can develop sufficient pressure during the mixing steps to push the mat cap from the tubes which can result in the transfer of droplets between tubes. The addition of the aluminum block to weight the mat cap onto the tubes, insures this cross-contamination does not occur. A second factor involved in limiting cross-contamination was the dispensing depths for the Hamilton pipette tips in the receiving tubes. The Hamilton pipette tips were set within 1 to 2 mm from the bottom of the tube for dispensing the standards, QCs, and internal standard. It was found that placing the tips close to the bottom of the tube helped prevent cross-contamination presumably caused by aerosol formation. Following these precautions, the percent cross-contamination observed was typically in the range of 0.025% or less.

3.9. Accuracy and precision—analysis of QC samples

The accuracy and precision data for the LC–MS–MS analysis of blank human plasma spiked with DEX and DOR at various levels and analyzed on 3 separate days are presented in Table 3. The average accuracy for DEX ranged from 92 to 104% with RSD values between 2.3 to 6.7% across the QC levels. For DOR, the average accuracy ranged from 93.0 to 101.4% with RSD values ranging from 3.2% to 9.2% across the QC levels.

Table 3
Accuracy and precision of the dextromethorphan and dextrorphan methodology ($n=24$)

Spiked (pg/ml)	Accuracy (RSD) (%)		
	Day 1	Day 2	Day 3
[DEX]			
131.2	98.5 (5.6)	100.4 (3.6)	96.3 (5.7)
1312	100.9 (2.3)	103.7 (2.4)	104.2 (6.7)
13 120	95.7 (4.2)	93.7 (2.5)	92.7 (4.8)
[DOR]			
109.2	99.2 (9.2)	96.4 (4.5)	93.0 (4.4)
1092	99.7 (8.2)	97.1 (3.9)	94.7 (3.2)
10 920	101.4 (4.6)	98.6 (5.5)	93.1 (5.4)

3.10. Ruggedness

The speed of the sample preparation process and the short LC–MS–MS analysis times makes it possible to run 400 samples, for both analytes, in a single day. The ruggedness of the entire method was therefore examined by performing over 1000 injections of standards and QC samples over a several day period. The peak shape of DEX and DOR remained unchanged up to 900 injections. However, slight tailing of the peak was observed between 900 and 1000 injections and it would be recommended to change the column after 1000 injections. During this period the back calculated values for all DEX and DOR standards were accurate within 100±15% and the accuracy and precision of the method as determined by the analysis of QC samples is provided in Section 3.9.

3.11. Human pharmacokinetic profiles following oral dosing of DEX

Plots of measured DEX and DOR plasma levels versus post-dose time interval for two human subjects orally dosed with 30 mg DEX/HBr are shown in Fig. 4. One subject was a fast metabolizer (Fig.

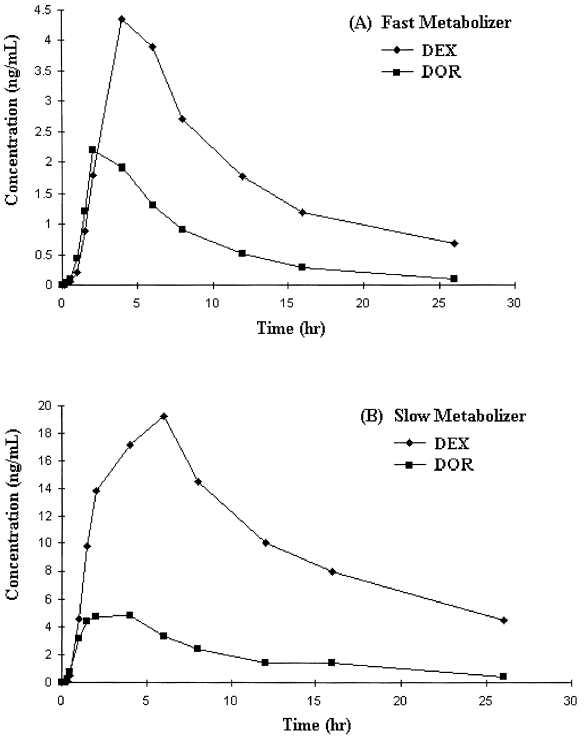

Fig. 4. Plots of plasma concentration of DEX and DOR versus post-dose collection time obtained from human subjects: (A) fast metabolizer and (B) slow metabolizer dosed orally with 30 mg of DEX hydrobromide in a commercial cough formulation.

4A) while the second subject (Fig. 4B) was a slow metabolizer. The concentration of DEX increased steadily until maximum concentration was achieved and then slowly decreased for the duration of the sampling period. The peak plasma concentrations were ~4 h for the fast metabolizer and ~6 h for the slow metabolizer. DEX and DOR were detected and quantified over the 26 h sampling period.

4. Conclusions

Through the use of semi-automated liquid handling systems and a 96-well plate format, a rapid LLE back-extraction method was developed for the quantitation of DEX and DOR in human plasma by LC–MS–MS. The parallel LLE approach greatly simplified the preparation process and decreased the time required for sample preparation by four fold versus a previously used manual method. The plunger-in-tip design of the liquid handling system allowed the facile manipulation of the ether extracts without dripping or cross-contamination. The LLE back-extract approach provided clean extracts exhibiting little suppression and proved sensitive enough to allow for the detection of the analytes for a 26 h period following oral dosing. In order to achieve speed in the methodology only a single extraction was performed at each step resulting in a relatively low absolute recovery of the analytes. However, the incorporation of the stable isotope-internal standards corrected for all analyte losses. The overall approach was shown to provide an accurate and precise methodology for the analysis of DEX and DOR in human plasma samples.

References

[1] J.P. Allanson, R.A. Biddlecombe, A.E. Jones, S. Pleasance, Rapid Commun. Mass Spectrom. 10 (1996) 811.
[2] J. Janiszewski, R. Schneider, K. Hoffmaster, M. Swyden, H. Fouda, Rapid Commun. Mass Spectrom. 11 (1997) 1033.
[3] B. Kaye, W.J. Herron, P.V. Macrae, S. Robinson, D.A. Stopher, R.F. Vern, W. Wild, Anal. Chem. 68 (1996) 1658.
[4] Y. Cheng, U.D. Neue, L. Bean, J. Chromatogr. A 828 (1998) 273.
[5] I.D. Davies, J.P. Allanson, R.C. Causon, J. Chromatogr. B 732 (1999) 173.
[6] M. Jemal, M. Haung, Y. Mao, D. Whigan, A. Schuster, Rapid Commun. Mass Spectrom. 14 (2000) 1023.
[7] S. Steinborner, J. Henion, Anal. Chem. 71 (1999) 2340.
[8] J. Zweigenbaum, K. Heinig, S. Steinborner, T. Wachs, J. Henion, Anal. Chem. 71 (1999) 2294.
[9] M. Jemal, D. Teitz, Z. Ouyang, S. Khan, J. Chromatogr. B 732 (1999) 501.
[10] N. Zhang, K.L. Hoffman, W. Li, D.T. Rossi, J. Pharm. Biomed. Anal. 22 (2000) 131.
[11] T.H. Eichhold, L.J. Greenfield, S.H. Hoke II, K.R. Wehmeyer, J. Mass Spectrom. 32 (1997) 1205.