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Simultaneous determination of dextromethorphan, dextrorphan, and guaifenesin in human plasma using semi-automated liquid/liquid extraction and gradient liquid chromatography tandem mass spectrometry

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Abstract

A method for the simultaneous determination of dextromethorphan (DEX), dextrorphan (DET), and guaifenesin (GG) in human plasma was developed, validated, and applied to determine plasma concentrations of these compounds in samples from six clinical pharmacokinetic (PK) studies. Semi-automated liquid handling systems were used to perform the majority of the sample manipulation including liquid/liquid extraction (LLE) of the analytes from human plasma. Stable-isotope-labeled analogues were utilized as internal standards (ISTDs) for each analyte to facilitate accurate and precise quantification. Extracts were analyzed using gradient liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). Use of semi-automated LLE with LC–MS/MS proved to be a very rugged and reliable approach for analysis of more than 6200 clinical study samples. The lower limit of quantification was validated at 0.010, 0.010, and 1.0 ng/mL of plasma for DEX, DET, and GG, respectively. Accuracy and precision of quality control (QC) samples for all three analytes met FDA Guidance criteria of ±15% for average QC accuracy with coefficients of variation less than 15%. Data from the thorough evaluation of the method during development, validation, and application are presented to characterize selectivity, linearity, over-range sample analysis, accuracy, precision, autosampler carry-over, ruggedness, extraction efficiency, ionization suppression, and stability. Pharmacokinetic data are also provided to illustrate improvements in systemic drug and metabolite concentration–time profiles that were achieved by formulation optimization.
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1. Introduction

To develop safe cough/cold products that deliver optimal efficacy, it is necessary to design formulations that provide the most favorable plasma concentration–time profiles of each active ingredient along with key metabolites. Two common actives in such products are dextromethorphan (DEX) and guaifenesin (GG). DEX is an antitussive which acts through depression of the medullary centers of the brain to decrease the involuntary urge to cough [1–5]. Guaifenesin is an expectorant believed to stimulate receptors that initiate a reflex secretion of respiratory tract fluid, thereby increasing the volume while decreasing the viscosity of mucus in the lungs. This action facilitates removal of mucus and reduces irritation of the bronchial tissue [6–8].

When evaluating the plasma concentration–time profiles of DEX, consideration must be given to a well-known phenotypic variation. In approximately 90% of the population, DEX undergoes a high degree of first-pass metabolism where the cytochrome P450 (CYP) 2D6 enzyme catalyzes *O*-demethylation to form dextrorphan (DET) [9]. This metabolite undergoes subsequent conjugation with glucuronide and is excreted in the urine [10]. Due to the high degree of first-pass metabolism, members of this group are consequently referred

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to as extensive metabolizers. The remainder of the population eliminates DEX more slowly and is classified in the poor metabolizer category. With poor metabolizers, there is a lesser degree of first-pass metabolism leading to markedly different pharmacokinetics when comparing profiles from the two populations. Poor metabolizers exhibit higher levels of circulating DEX and have much longer elimination half-lives compared with extensive metabolizers. Half-lives of slow metabolizers reportedly range from 17 to 22 h versus 1 to 4 h for extensive metabolizers [11].

While there are several known metabolites of DEX [12,13], the parent drug and its unconjugated metabolite, DET, are the two molecules primarily responsible for antitussive activity [14–16]. Therefore, it is of interest to measure the plasma concentrations of both compounds to assess the relationship between concentration and antitussive response.

Unlike dextromethorphan, guaifenesin is not subject to phenotypic variation. Following oral administration, guaifenesin is rapidly absorbed and is excreted primarily in the urine either unchanged or after metabolism to β-(2-methoxy-phenoxy)lactic acid. There are no known active metabolites of GG [17–19].

Optimized cough/cold formulations containing DEX or a combination of DEX and GG were developed through a series of iterative, rapid learning studies using plasma concentration measurements as a key parameter in evaluating relative performance [20]. The optimized formulations were then tested in more rigorous studies involving single- and multiple-dose clinical pharmacokinetic investigations to fully characterize selected products containing DEX or DEX and GG.

When considering multiple analytes with diverse functionality and the overall project objectives, the specific measurement requirements presented a unique analytical challenge. Providing adequate characterization of plasma concentration–time profiles necessitated relatively low limits of quantification (LLOQ) with target levels at 10 pg/mL of plasma for DEX and DET and 1 ng/mL for GG. Also, an extensive quantitative range was needed for each analyte, with a target of 5000 times the LLOQ. This range was defined by the need to generate PK profiles from single- and multiple-dose studies that contained both poor and extensive DEX metabolizers, as well as multiple formulations that were designed to alter the rate of drug delivery. With more than 6200 samples for analysis, additional method objectives included a single assay for simultaneous quantification of all three analytes, a high degree of automated sample handling, and a rapid rate of sample throughput for both preparation and analysis.

Several analytical approaches for measuring human plasma levels of DEX and/or DET have been reported in the literature. Most of these methods are based on chromatographic separations and utilize various detection schemes including LC-fluorescence [21–24], LC–UV [25], CE–UV [26], and GC–NPD [27,28]. In recent years, several liquid chromatographic based assays were reportedly used in combination with tandem mass spectrometry (LC–MS/MS). Sample preparation for the LC–MS/MS methods include liquid/liquid extraction [29,30], solid phase extraction [31], and, for analysis in rat plasma, the use of on-line turbulent flow chromatography [32]. There is far less published quantitative bioanalytical methodology for GG than for DEX. Early methods for quantification of GG in human plasma were based on LC–UV [17,18] and GC–ECD following analyte derivatization [33]. A recently reported method based on LC–MS/MS demonstrated faster sample analyses and lower quantification limits for determination of GG in human plasma [34].

Few methods have been reported for the quantification of DEX and/or DET simultaneously with GG in human plasma. An LC-fluorescence method was used for the quantification of both total DET and GG in plasma [35]. However, DEX was not included as an analyte with this method and the reported LLOQs of 20 and 180 ng/mL of plasma for DET and GG, respectively, are not nearly low enough to adequately define the concentration–time profiles in the current studies. A recently reported on-line solid phase extraction (SPE) combined with MS/MS detection represented a significant advance in the simultaneous determination of these compounds [36]. The reported LLOQ for DEX and unconjugated DET was 50 pg/mL, while for GG, the LLOQ decreased to 5 ng/mL. However, the sample cleanup procedure limited the ultimate performance of this method, especially with regard to sensitivity, ruggedness, and reliability. As described in further detail below, this approach is unable to meet the demanding requirements of the current application.

To achieve the stated quantification limits of the on-line SPE method, a maximum plasma sample volume of 100 μL was centrifuged and then filtered prior to injection. Even with relatively small plasma volumes, there were problems with plugging, which limited the utility of this method for overnight analysis of large batches of samples. Additionally, the analysis of plasma blanks produced some chemical noise in the background; this was especially evident for GG. When considering the limitation of using relatively small volumes of plasma and the chemical noise observed in the background, the attainable LLOQs were 5-fold higher than the requirements of the current assay. The nature of these limitations also indicated that using a more sensitive triple quadrupole would not allow a commensurate decrease in quantification limits.

While the process of optimizing conditions for the on-line SPE is automated, when developing an assay requiring quantification of structurally diverse compounds, it is very challenging to achieve acceptable recoveries of all analytes from the SPE cartridge under conditions that also allow adequate focusing of all analytes on the analytical column. The optimal conditions for these analytes did produce relatively good peak shapes for DEX and DET, but the GG peak shape suffered from tailing. Using the optimal conditions for sequential extraction and analysis limited throughput as a relatively long injection-to-injection time of 9 min per sample was required, resulting in an analysis time of 14.4 h per 96-well plate. Certainly faster analyses could be achieved by simultaneously performing the extraction and the LC separation; however, this approach also increases complexity of the experimental setup. The relatively long analysis time

and the potential for failures due to plugging both combined to limit sample throughput as well as ruggedness and reliability. Also, with the on-line SPE method, there is a high on-going cost of expendables at US$ 320 per 96-well extraction tray along with a relatively high replacement rate of analytical and guard columns.

While the on-line SPE method does provide an adequate solution for smaller batches of samples where higher LLOQs are acceptable, it is not a practical solution when considering the demanding needs of the current assay requiring simultaneous ultratrace quantification of these three structurally diverse analytes in more than 6200 study samples. The relatively clean plasma extracts produced by LLE directly address many of the performance issues of the on-line SPE approach. Cleaner extracts allow the use of larger sample volumes while providing lower background chemical noise and lower detection limits. Cleaner extracts also greatly improve reliability and ruggedness, and allow the potential for higher sample throughput. These factors along with the reduced cost of expendables make the liquid/liquid extraction an attractive approach to sample preparation when developing challenging assays for use with extensive PK investigations. Details of the LLE and LC–MS/MS method development are presented along with data from the thorough characterization of the assay during validation and analysis of over 6200 samples. These data address selectivity, linearity, over-range sample analysis, accuracy, precision, autosampler carry-over, ruggedness, extraction efficiency, ionization suppression, and analyte stability. Examples of pharmacokinetic data are also provided that demonstrate plasma concentration–time profile improvements resulting from formulation optimization.

2. Experimental

2.1. Chemicals and reagents

The analytes and their respective stable-labeled analogues were obtained from the following sources: dextromethorphan·hydrobromide was purchased from Hoffman/LaRoche; [$^{2}H_3$-*O*-methoxy]-dextromethorphan was synthesized at The Procter and Gamble Co., Ross, OH; dextrorphan·D-tartrate was purchased from Sigma Chemical Co., St. Louis, MO; [$^{13}C_1$, $^{2}H_3$-*N*-methyl]-dextrorphan was synthesized at Procter & Gamble Pharmaceuticals, Norwich, NY; guaifenesin was obtained from the USP (Lot G), Rockville, MD; and [$^{13}C_3$]-guaifenesin was supplied by Isotec Inc., Miamisburg, OH.

Deionized (DI) water used for both sample preparation and LC–MS/MS analysis was obtained from a Barnstead Nano-Pure system (Dubuque, IA). The HPLC grade methanol used for sample preparation and chromatographic mobile phases was obtained from J.T. Baker, Inc., Phillipsburg, NJ. Ethyl ether, sodium hydrogen carbonate, sodium carbonate, sodium chloride, and formic acid (88%) were also purchased from J.T. Baker, Inc. Carbonate buffer, 1 M, was prepared by adding 31.8 g of Na_2CO_3 and 16.8 g of $NaHCO_3$ to 500 mL DI water and stirring until dissolved. Normal human plasma obtained from Golden West Biologicals (Temecula, CA) was used for preparation of calibration standards, quality control (QC) samples, and plasma blanks.

2.2. Instrumentation and materials for sample handling

A MicroLab AT plus 2 (Hamilton Co., Reno, NV) was used to perform liquid transfers during LLE. This liquid-handling device facilitated the transfer of plasma from individual 2 mL polypropylene cryovials (Sarstedt, Inc., Newton, NC) into the 96-well format. It was also used for addition of the internal standard (ISTD) solution, carbonate buffer, and ethyl ether as described in detail below.

Two different types of 96-well plates were used to process the samples. One type of plate was used during the ethyl ether extraction because of its superior sealing properties and ability to contain volatile solvents, while the second type of plate was more compatible with the HPLC autosampler. The LLE was performed in a 1.2 mL micro-tube cluster plate (Abgene, Inc., Rochester, NY, P/N AB-0595) which was sealed with cap mats obtained from Matrix Technologies Corporation (Hudson, NH, P/N 4431). In order to minimize potential for well-to-well contamination and to assure a good phase separation during the extraction process, plates were briefly centrifuged using a Hermle Model Z 360 K centrifuge (National Labnet Co., Woodbridge, NJ). Following centrifugation, the MicroLab was used to transfer a portion of organic layer into a 1.2 mL, 96-deep-well plate (Greiner Bio-One, Longwood, FL, P/N 780201).

The ethyl ether was evaporated using a SPE Dry-96 solvent evaporator (Jones Chromatography, Lakewood, CO) with nitrogen gas warmed to 30 °C. A 96-channel pipettor, the Multimek 96 (Beckman Coulter, Fullerton, CA), was used to add 150 μL of 1% formic acid to each well for sample reconstitution. The plate was then sealed with a teflon/silicon cap mat (Sun International, Wilmington, NC, P/N 400067).

2.3. Preparation of solutions for calibration and quality control

A stock solution of the analytes was prepared in water–methanol (75:25, v/v) at nominal concentrations of 500/500/50,000 ng/mL of DEX/DET/GG. Spiking solutions were then prepared by diluting the stock solution in water–methanol (75:25, v/v) with 0.1% sodium chloride to form twelve solutions. Plasma standards were prepared by adding 20 μL of each of the twelve spiking solutions to 200 μL portions of human plasma using the Hamilton Microlab AT Plus 2. This process yielded calibration standards with nominal concentrations of 0.01/0.01/1, 0.025/0.025/2.5, 0.05/0.05/5, 0.1/0.1/10, 0.25/0.25/25, 0.5/0.5/50, 1/1/100, 2.5/2.5/250, 5/5/500, 10/10/1000, 25/25/2500, and 50/50/5000 ng/mL of DEX/DET/GG in human plasma, respectively.

Quality control samples were prepared manually in bulk quantity and 1.5 mL portions were transferred to 2 mL Sarstedt polypropylene cryovials. These vials were stored frozen at −70 °C until the time of analysis. QC samples were prepared at levels of 0.01/0.01/1.0 (for validation only), 0.03/0.03/3.0,

1/1/10, 20/20/2000, and 40/40/4000 ng/mL for DEX/DET/GG, respectively.

2.4. Plasma sample preparation and liquid/liquid extraction

Study samples, QC samples, and blank plasma were removed from the freezer and allowed to reach room temperature. The contents of all containers were mixed thoroughly by vortexing. Prior to starting the MicroLab program, 200 μL blank plasma aliquots were transferred to positions in a 96-well plate designated for plasma blank, zero standard, or calibration standard using a repeater pipette. QC samples, study samples, and standard spiking solutions were stored in 2 mL cryovials which were placed in predetermined positions on the primary sample rack. Internal standard solution, carbonate buffer, and ethyl ether were sampled from their respective reagent containers which were also placed in designated positions on the MicroLab platform. The MicroLab then transferred spiking solutions, samples, and reagents to the extraction plate in the following order: (1) for calibration standards, 20 μL aliquots of standard spiking solutions were added to blank plasma in their respective positions; (2) 200 μL aliquots of QC samples and study samples were transferred to their assigned positions on the extraction plate; (3) 20 μL aliquots of ISTD spiking solution were added to all wells except those designated for blanks or autosampler wash; (4) 50 μL of carbonate buffer was added to each well; (5) a total of 0.6 mL of ethyl ether was added in two 0.3 mL increments.

After addition of all reagents, the plate was removed from the MicroLab platform, sealed with a cap mat and mixed using a vortex mixer for 3 min. The extraction plate was centrifuged for 2 min at 1000 rpm to remove residual solvent from the cap mat and assure phase separation. The plate was then returned to the MicroLab platform where 0.3 mL of the ethyl ether extracts were transferred to corresponding wells corresponding well of a new Masterblock 96-well plate. Care was taken to assure that ether was sampled well above the phase interface because contamination of the ether extract with the aqueous layer resulted in detrimental effects on ruggedness, chromatographic peak shape, and ionization. The ethyl ether was evaporated under a stream of nitrogen using the 96-well plate dryer and the sample residues in each well of the plate were simultaneously reconstituted with 150 μL of 1% formic acid using the Beckman Multimek 96-channel pipettor. The contents were mixed and the plate was ready for LC–MS/MS analysis.

2.5. Instrumentation for LC–MS/MS analysis

The HPLC was comprised of a ternary pumping system including a model 305 controller pump and two 306 auxiliary pumps equipped with a model 805 manometric module, and a 65 μL mixing chamber (Gilson, Middletown, WI). The HPLC autosampler was an HTS PAL (CTC Analytics AG, Switzerland) equipped with a Peltier cooled tray holder and a three drawer stack with capacity to hold up to six 96-well plates. The mass spectrometer was an API 3000 triple quadrupole (AB/MDS Sciex, Thornhill, ON, Canada) and quantitative data analysis was performed using the MacQuan software package, Version 1.6.

2.6. LC–MS/MS conditions

A portion (2–50 μL) of each reconstituted plasma extract was introduced onto a 2.1 mm × 30 mm XTerra MS C18 column (Waters, Inc., Milford, MA), equipped with a 2.1 mm × 10 mm guard column with the same stationary phase. The weak mobile phase (MP) consisted of water containing 0.1% formic acid and the strong MP was methanol with 0.1% formic acid. The column was equilibrated 2 min prior to injection with 80:20 (weak:strong) and this mixture was held constant for 0.25 min following injection of a plasma extract. A rapid 0.75 min linear gradient was then performed resulting in a final MP composition of 30:70 (weak:strong) followed by a final hold of 0.25 min, after which the composition was returned to initial conditions. A flow rate of 0.35 mL/min was maintained throughout. MS/MS data were collected for 2.5 min, after which time the autosampler began to load the subsequent sample. Column re-equilibration occurred for 2 min prior to performing the next injection. The total injection-to-injection cycle time was 3.25 min.

The autosampler used two solvents for syringe washing between sample injections. The first consisted of water with the second being a mixture of water–methanol–formic acid (75:25:0.05, v/v/v). The autosampler syringe was used to rinse the valve three times with 100 μL portions of each solvent between injections.

The mass spectrometer was operated in the turbo ionspray, positive ion mode. This configuration consisted of an articulated ionspray inlet used in conjunction with the heated TurboProbe desolvation unit. The TurboProbe temperature and nitrogen gas flow rate were 450 °C, and 8 L/min, respectively. Collisional activation was achieved using nitrogen as the target gas, at a thickness of 2.7×10^{15} molecules cm^{-2}. A collision energy of 33 eV was used for DEX and DET activation, while 13 eV was used for GG. The following MS/MS transitions were monitored for quantification: DEX, *m/z* 272 to 215; DEX ISTD, *m/z* 275 to 218; DET, *m/z* 258 to 201; DET ISTD, *m/z* 262 to 201; GG, *m/z* 199 to 151; GG ISTD, *m/z* 202 to 153.

2.7. Selectivity and lower limit of quantification

To evaluate method specificity, blank human plasma obtained from six different subjects was prepared, analyzed, and examined for response in each of the analyte and ISTD chromatographic profiles. The LLOQ was established at a level for which the response was greater than five times the blank response. The accuracy and precision criteria required that the LLOQ calibration standard and QC accuracies average within 20% of target with a CV of ≤20% for five validation batches.

2.8. Linear dynamic range and over-range samples

Method linearity was investigated by analyzing a set of calibration standards with each of the five validation batches. Calibration curves were constructed by plotting area ratios (analyte/ISTD) versus analyte concentrations for the 12 calibration standards and performing a weighted, $1/x^2$ linear regression analysis. Linearity was evaluated by examining the correlation

coefficients of the calibration curves and by determining the accuracy of the calibration standards when calculating their concentrations using the regression curve parameters. Accuracy was determined by dividing the measured concentration by the theoretical concentration and multiplying by 100 to express the value as a percentage.

With samples collected from poor metabolizers during multiple-dose studies, some analyte concentrations in human plasma were expected to exceed the upper limit of quantification (ULOQ), especially in the case of DEX. To account for these high analyte concentrations, a 10-fold plasma dilution procedure was validated using plasma QC samples prepared at concentrations five times higher than the upper limits of the linear curves (250/250/25,000 ng/mL for DEX/DET/GG). Triplicate 20-μL aliquots of the over-range QC samples were added to 180 μL of blank plasma, which were then processed and analyzed. A 10-fold dilution factor was applied to the resulting concentration that was quantified versus the normal calibration curve.

2.9. Injection volume and signal saturation

The method performance using variable injection volumes was examined at each calibration standard concentration by injecting each standard plasma extract at volumes of 2, 5, 10, and 50 μL. The resulting area ratios (analyte/ISTD) for the calibration standards were plotted on a single calibration curve for all injection volumes of each analyte. Injection volumes providing acceptable quantitative data were identified based on the following criteria: both analyte and ISTD signal-to-noise ratios were greater than 5:1; there was no signal saturation for analyte or ISTD; and the accuracies of standards as calculated by the calibration curve algorithm were typically within 7% of target. Signal saturation was evaluated by determining analyte intensity at which the analyte to ISTD area ratio consistently resulted in a negative deviation from the other points on the calibration curve and resulted in accuracy calculations that were lower than 7% of the target level.

Using the data from this investigation, 50 μL injection volumes were typically used for analyses of blanks, standards, QCs, and study samples. However, in cases where the analyte response exceeded the point of signal saturation, the injection volume was reduced, usually to 5 μL. When using a 50 μL injection volume, saturation was typically observed with high QC samples, the three highest calibration standards, and study samples with analyte concentrations in the same range. Using these injection conditions for constructing the calibration curve, three aliquots of plasma spiked at levels of 1/1/100 ng/mL DEX/DET/GG were prepared and analyzed with injection volumes of 2, 5, 10, 20, and 50 μL. The accuracies were back calculated to demonstrate consistent quantitative accuracy, regardless of injection volume.

2.10. Accuracy and precision

During method validation, QC samples were prepared by spiking known amounts of DEX/DET/GG into normal human plasma at four distinct levels. These levels were: LLOQ QC, 0.01/0.01/1.0; low QC, 0.03/0.03/3.0; mid QC, 1/1/100; and high QC, 40/40/4000 ng/mL plasma for DEX/DET/GG, respectively. Accuracy and precision were determined during analysis of five validation batches, with each batch containing six replicates at each QC level. The accuracy and precision criteria required that for QCs above the LLOQ, accuracies must average within 15% of target with a CV of ≤15% for five validation batches (see Section 2.7. for LLOQ QC criteria).

Table 1
Summary of clinical studies, numbers of plasma samples, and analytes quantified for each study

Study number	Samples analyzed	Analytes
1	664	DEX, DET
2	664	DEX, DET, GG
3	1044	DEX, DET
4	539	DEX, DET
5	1320	DEX, DET
6	1978	DEX, DET, GG
Total	6209	

Throughout the course of clinical sample analysis (see Table 1), accuracy and precision of the assay were continually monitored with QC samples prepared at three levels. For each 96-well plate containing study samples, QC samples were included at the low, mid, and high levels previously described, with three replicates at each level per plate. For studies 4–6, an alternate high QC concentration was used at 20/20/2000 ng/mL for DEX/DET/GG.

2.11. Autosampler carry-over

Immediately following a 5 μL injection of the highest calibration standard containing 500/500/50,000 ng/mL DEX/DET/GG, three 50 μL injections of a 1% formic acid blank were sequentially performed. All analyte and internal standard peak windows were examined for the presence of measurable response due to carry-over. Peaks were integrated and the percent carry-over was computed by dividing these areas by the corresponding peak area produced upon injection of the highest calibration standard.

2.12. Batch size and ruggedness

During validation experiments, two 96-well plates were used to evaluate the capability of the LC–MS/MS method to produce quality data over a multi-plate batch. The first plate contained a set of 12 calibration standards and triplicate aliquots of low, mid, and high level QC samples. Blank plasma was added to the remaining wells on the plate. The second plate contained six replicates of the low, mid, and high QC samples with blank plasma aliquoted into the remaining wells. Both plates were subjected to the semi-automated LLE procedure and all wells on both plates were injected under LC–MS/MS method conditions, in duplicate, to simulate analysis of a batch consisting of four 96-well plates. Method sensitivity, quantitative accuracy, and column back pressure were monitored to determine the capability of the LC–MS/MS method to successfully produce quality data during analysis of batches comprised of up to 384 plasma extracts.

2.13. Extraction efficiency and ionization suppression

Analyte and internal standard extraction efficiencies from human plasma were determined by comparing the LC–MS/MS response for each analyte and internal standard when analyzing plasma samples spiked with all six compounds prior to extraction versus the response obtained when the compounds were added to a prepared blank plasma matrix after extraction and immediately prior to reconstitution. Three replicates were spiked at the low, mid, and high QC levels with the six compounds of interest before and after LLE.

Signal loss from all sources, including ionization suppression and extraction from plasma, was determined by directly comparing the LC–MS/MS responses for analytes and internal standards produced from the analysis of prepared, spiked plasma samples with the corresponding responses obtained from injection of all six compounds in neat solutions of 1% formic acid. The mean response for each compound in plasma samples spiked at low, mid, and high QC levels was determined from six replicates, while each corresponding mean response was determined in triplicate from neat solutions.

2.14. Stability

Stability of DEX, DET, and GG was determined under a variety of storage conditions chosen to simulate those expected to be encountered during the collection, storage, and analysis of study samples. Analyte stability was evaluated during: refrigeration of standard spiking solutions for 34 days; storage of whole blood for 1 and 2 h at room temperature; exposure of plasma to room temperature on the bench top for 2 and 6 h; freezing and thawing of plasma up to three times; storage of plasma at −70 °C for 55 days, 6 months, and 1 year; and refrigeration of reconstituted extracts for 12 days. For stability studies, QC concentrations of 0.03/0.03/3.0 and 40/40/4000 ng/mL for DEX/DET/GG, respectively, were used for evaluation of most storage conditions, except as noted below. A minimum of three replicate measurements were performed at each level and recoveries were determined versus a freshly prepared calibration curve. The exception to this was the stability evaluation of the reconstituted extracts which were quantified using the original calibration curve, as the prepared plates already contained internal standard.

Stability of DEX, DET, and GG spiking solutions was determined so that weighing and dilution of standards was not required each day of sample analysis. Spiking solutions for calibration standards 3 and 11 were prepared in water–methanol (75:25, v/v) with 0.1% sodium chloride and stored in Sarstedt polypropylene cryovials at 4 °C for 34 days. At the end of 34 days, these solutions were used to spike plasma samples in triplicate, which were then quantified versus a freshly prepared calibration curve using freshly weighed standards.

For determining stability in fresh whole blood, 5 mL aliquots of human blood were spiked at 0.1/0.1/10 and 5/5/500 ng/mL DEX/DET/GG. Plasma was isolated from the samples immediately after spiking and following intervals of 1 and 2 h of storage at room temperature. Sample preparation was initiated by centrifuging the spiked blood samples for 10 min at 3500 rpm to separate the plasma from the cells. The plasma was transferred to cryovials and triplicate 200 μL portions of each sample were prepared for analysis according to the described methodology. Stability was determined by comparing the recoveries obtained after 1 and 2 h of storage versus that which was determined following immediate processing of spiked blood.

For determining analyte stability in plasma, triplicate samples were spiked at the low and high QC levels for DEX, DET, and GG and subjected to a range of storage conditions. To assure stability on the bench top in plasma prior to extraction, stability samples were stored in 2 mL Sarstedt cryovials at room temperature for 2 and 6 h. For determining long-term stability in frozen plasma, samples were placed in Sarstedt cryovials and stored at −70 °C for periods of 55 days, 6 months, and 1 year. To simulate multiple samplings from a given cryovial, stability samples were subjected to three freeze/thaw cycles. Within each cycle, samples were frozen at −70 °C for a minimum of 12 h and then brought to room temperature without additional heat from any source other than exposure to ambient room conditions.

Post preparation sample stability was investigated to provide some flexibility in cases where immediate analysis of sample extracts was not possible. This was investigated by the reanalysis of a single plate from one of the validation batches containing a set of calibration standards and six replicates of each QC sample. After the initial analysis, the plate was removed from the autosampler and placed in a refrigerator at 4 °C. Following an interval of 12 days, the calibration standards and QC samples were reanalyzed and the recoveries of the low and high QCs were determined for all six samples.

2.15. Pharmacokinetic study

A clinical study was conducted to compare the pharmacokinetics of DEX, unconjugated DET, and GG in twenty extensive and eight poor metabolizers following a single dose of two products containing DEX and GG. This was an investigator blind, randomized, cross-over study comparing a conventional formulation with an optimized formulation. The conventional formulation was Robitussin DM (Wyeth, Madison, NJ). A 10 mL dose containing 20 mg DEX HBr and 200 mg GG was administered orally. The optimized formulation contained an equivalent amount of DEX and GG. Blood samples were collected in sodium heparin tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) from all subjects prior to dosing and then at 0.05, 0.17, 0.33, 0.67, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, and 24 h post dosing. For slow metabolizers additional blood samples were taken at 48, 72, 120, and 168 h post-dosing. After collection, blood was centrifuged and the plasma was harvested and stored frozen at −70 °C in polypropylene cryovials until the time of analysis.

3. Results and discussion

The method for quantification of DEX, DET, and GG in human plasma was validated using the described procedures based on the Federal Guidance for Industrial Bioanalytical

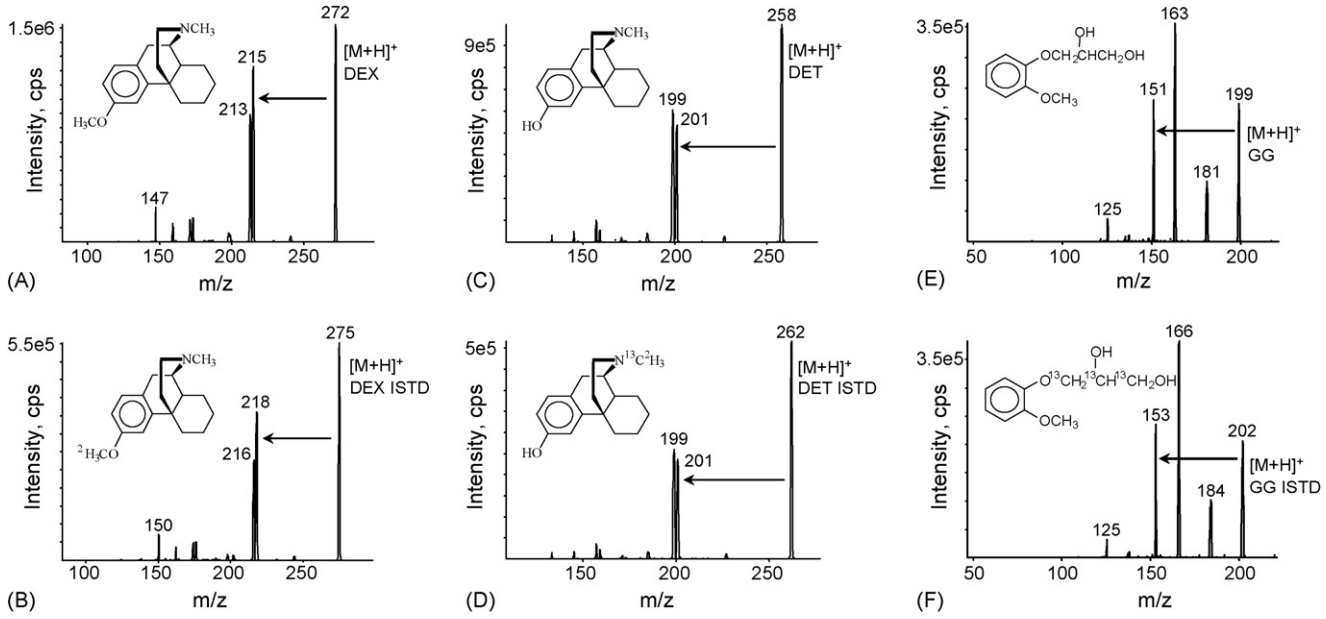

Fig. 1. Full-scan, product ion spectra of (A) DEX, (B) DEX ISTD, (C) DET, (D) DET ISTD, (E) GG, and (F) GG ISTD recorded under dissociation conditions used for quantification. Arrows indicate the transitions monitored to generate chromatographic profiles. Chemical structures for each analyte and internal standard are also shown as well as label positions for the internals standards.

Method Validation [37], prior to analysis of study samples. The results of method characterization during validation follow. Additionally, key performance data generated during method application to six clinical PK studies that produced more than 6200 plasma samples are summarized. An overview of these studies is captured in Table 1, including the number of samples and the target analytes for each study. Examples of PK results are also provided and discussed.

3.1. Product ion spectra and chromatography

The chemical structures of DEX, DET, and GG and their three corresponding stable-labeled internal standards are displayed in Fig. 1 along with the respective product ion mass spectra. These spectra were collected during infusion of the analytes under collision conditions used for quantification, with arrows indicating the selected precursor to product ion transitions monitored with the assay.

For the simultaneous determination of DEX, DET, and GG, gradient chromatographic conditions were developed to add specificity to the assay and also to elute all three analytes with good peak shapes in a reasonably short time scale. All three compounds eluted between 1 and 2 min after injection. LC–MS/MS data were collected through 2.5 min and the total injection-to-injection cycle time was 3.25 min. Typical peak shapes and retention times of the SRM chromatograms are shown in Fig. 2.

3.2. Selectivity and lower limit of quantification

During validation, analysis of six individual sources of normal human plasma produced no response for GG or any of the stable-labeled internal standards. One of the six plasma sources did result in quantifiable responses for DEX and DET. Since both drug and metabolite were observed in the same source of purchased plasma, it is likely that the individual had taken a dose of OTC medication containing DEX prior to donating blood used to produce the plasma. Throughout clinical study sample analysis, pre-dose samples did not generate a significant response for any of the analytes without an assignable cause such as contamination or insufficient wash-out period from previous dosage of medication, as is particularly possible with multiple-dose studies when sampling poor DEX metabolizers.

The LLOQ for the method was established at 0.010/0.010/1.0 ng/mL plasma for DEX/DET/GG, respectively. Typical SRM chromatograms produced by injection of a plasma blank spiked with the three internal standards are shown in Fig. 2(A). For comparison, Fig. 2(B) contains ion traces of the same six transitions produced by injection of an LLOQ calibration standard, showing typical analyte signal-to-noise ratios achieved at the LLOQ. Analysis of study samples produced similar chromatographic results with no additional peaks observed. Accuracy and precision for the assay at the LLOQ levels measured during validation are shown in Tables 2–4 for the calibration standards and in Tables 5–7 for the QC samples. These results indicate excellent accuracy and precision, as these data easily exceeded the LLOQ guidance accuracy criteria of ±20% with target CV values of ≤20%.

3.3. Linear dynamic range and over-range samples

These studies involved a wide range of expected plasma levels of DEX, DET, and GG due to the evaluation of several formula-

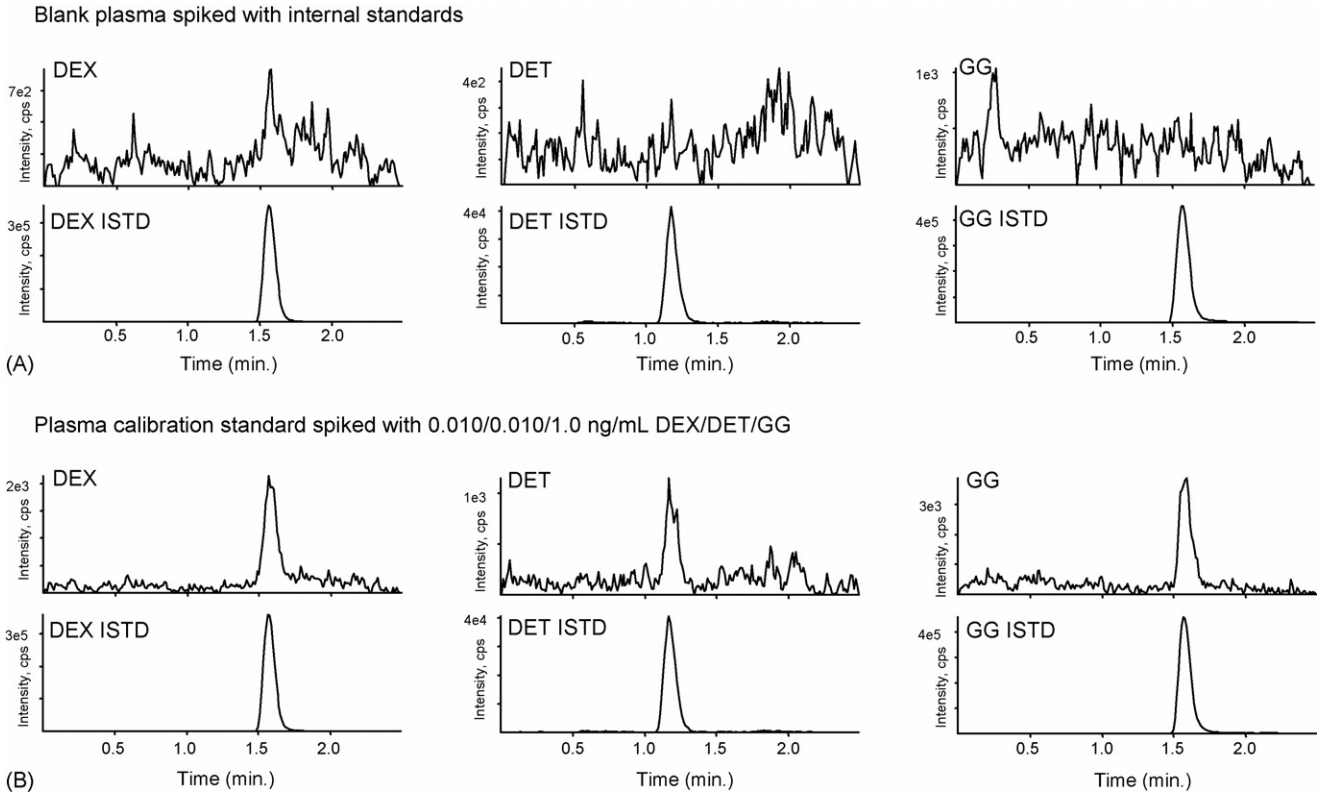

Fig. 2. Chromatograms generated during analysis of (A) blank plasma spiked with internal standards by monitoring transitions selective for DEX, DET, GG, and their corresponding internals standards. For comparison, the same conditions were used for analysis of (B) an LLOQ calibration standard.

Table 2
Accuracy of DEX calibration standards during assay validation

Batch number	Percent accuracy of DEX calibration standards											
	CS 1[a] 0.010[b]	CS 2[a] 0.025[b]	CS 3[a] 0.050[b]	CS 4[a] 0.10[b]	CS 5[a] 0.25[b]	CS 6[a] 0.50[b]	CS 7[a] 1.0[b]	CS 8[a] 2.5[b]	CS 9[a] 5.0[b]	CS 10[a] 10[b]	CS 11[a] 25[b]	CS 12[a] 50[b]
I	97.2	108.8	99.1	93.9	96.0	103.9	99.7	99.1	100.1	102.9	99.5	99.8
II	102.2	97.3	100.1	91.7	98.1	93.7	101.1	103.5	104.8	107.4	102.3	97.9
III	104.5	90.7	95.8	98.6	102.2	103.6	103.4	98.7	97.3	101.5	101.6	102.2
IV	101.5	97.4	95.7	103.3	101.7	98.7	101.0	100.2	100.6	101.7	95.7	102.5
V	98.3	102.1	101.4	103.5	103.9	103.6	102.9	100.4	96.9	97.1	95.7	94.1
Mean accuracy (%)	100.7	99.3	98.4	98.2	100.4	100.7	101.6	100.4	99.9	102.1	99.0	99.3
CV (%)	3.0	6.8	2.6	5.5	3.2	4.4	1.5	1.9	3.2	3.6	3.2	3.5

[a] Calibration standard.
[b] Concentration (ng/mL).

Table 3
Accuracy of DET calibration standards during assay validation

Batch number	Percent accuracy of DET calibration standards											
	CS 1[a] 0.010[b]	CS 2[a] 0.025[b]	CS 3[a] 0.050[b]	CS 4[a] 0.10[b]	CS 5[a] 0.25[b]	CS 6[a] 0.50[b]	CS 7[a] 1.0[b]	CS 8[a] 2.5[b]	CS 9[a] 5.0[b]	CS 10[a] 10[b]	CS 11[a] 25[b]	CS 12[a] 50[b]
I	103.7	93.5	95.3	98.1	99.8	98.3	99.8	106.5	100.9	96.3	103.9	103.9
II	102.0	98.9	95.1	92.3	106.6	92.4	98.3	103.4	107.6	106.3	95.9	101.2
III	94.4	112.3	103.9	99.0	101.8	97.5	95.4	94.1	92.8	105.6	100.8	102.3
IV	101.0	96.2	102.0	102.2	96.7	101.6	103.4	101.6	101.2	95.0	97.3	101.7
V	97.4	105.4	99.1	99.4	97.9	98.0	98.5	101.6	96.0	95.8	106.5	103.3
Mean accuracy (%)	99.7	101.3	99.1	98.2	100.6	97.6	99.1	101.4	99.7	99.8	100.9	102.5
CV (%)	3.8	7.5	4.0	3.7	3.9	3.4	2.9	4.5	5.7	5.7	4.4	1.1

[a] Calibration standard.
[b] Concentration (ng/mL).

Table 4
Accuracy of GG calibration standards during assay validation

Batch number	Percent accuracy of GG calibration standards											
	CS 1[a] 1.0[b]	CS 2[a] 2.5[b]	CS 3[a] 5.0[b]	CS 4[a] 10[b]	CS 5[a] 25[b]	CS 6[a] 50[b]	CS 7[a] 100[b]	CS 8[a] 250[b]	CS 9[a] 500[b]	CS 10[a] 1000[b]	CS 11[a] 2500[b]	CS 12[a] 5000[b]
I	101.3	97.5	98.2	101.7	99.7	98.6	98.4	98.9	96.7	103.0	105.1	100.9
II	105.6	91.9	95.4	88.3	96.4	93.4	101.5	105.6	98.1	113.8	107.0	103.0
III	103.0	95.0	97.3	94.5	99.8	101.9	106.7	101.8	105.1	108.0	92.6	94.4
IV	100.3	99.9	94.0	108.0	102.8	102.8	102.2	98.7	85.7	104.0	102.6	99.2
V	99.3	98.0	103.2	106.5	100.9	105.1	106.1	98.4	99.3	97.0	97.3	88.9
Mean accuracy (%)	101.9	96.5	97.6	99.8	99.9	100.4	103.0	100.7	97.0	105.2	100.9	97.3
CV (%)	2.4	3.2	3.6	8.3	2.3	4.5	3.3	3.1	7.3	5.9	5.9	5.8

[a] Calibration standard.
[b] Concentration (ng/mL).

Table 5
Accuracy and precision of DEX QC samples during validation and study sample analysis

Study	*n* QCs	LLOQ QC (0.010 ng/mL)		Low QC (0.030 ng/mL)		Mid QC (1.0 ng/mL)		High QC (40 ng/mL)	
		Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
Validation	30	101.8	12.7	98.9	7.6	104.0	3.9	97.1	5.8
1	39			103.5	8.7	101.4	2.5	89.5	3.6
2	39			103.1	6.9	103.8	4.1	92.6	5.5
3	72			101.7	10.6	96.8	3.2	88.2	4.9
4	30			102.6	8.3	101.3	2.7	96.1[a]	3.9
5	70			105.3	8.1	103.0	4.5	96.0[a]	7.7
6	102			100.0	7.9	100.1	3.5	93.1[a]	3.5
Totals	382	101.8	12.7	102.1	8.7	100.9	4.3	92.8	6.2

[a] 20 ng/mL.

Table 6
Accuracy and precision of DET QC samples during validation and study sample analysis

Study	*n* QCs	LLOQ QC (0.010 ng/mL)		Low QC (0.030 ng/mL)		Mid QC (1.0 ng/mL)		High QC (40 ng/mL)	
		Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
Validation	30	104.2	14.6	99.0	10.7	104.1	4.8	100.4	7.8
1	33			96.4	6.7	99.6	2.9	94.8	10.1
2	36			103.8	13.6	103.2	5.1	100.3	6.6
3	72			97.1	5.5	97.2	3.9	92.3	4.9
4	30			103.0	10.3	100.8	2.4	99.1[a]	4.4
5	70			100.2	9.0	104.3	4.8	103.3[a]	7.3
6	102			101.9	7.7	100.6	3.3	97.8[a]	5.1
Totals	373	104.2	14.6	100.2	9.1	101.1	4.7	98.1	7.4

[a] 20 ng/mL.

Table 7
Accuracy and precision of GG QC samples during validation and study sample analysis

Study	*n* QCs	LLOQ QC (1.0 ng/mL)		Low QC (3.0 ng/mL)		Mid QC (100 ng/mL)		High QC (4000 ng/mL)	
		Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
Validation	30	108.5	12.6	101.3	7.6	105.9	5.0	99.0	5.1
2	36			106.1	6.9	102.9	4.2	93.4	5.4
6	102			101.5	7.1	99.6	3.6	96.2[a]	5.5
Totals	168	108.5	12.6	102.4	7.4	101.4	4.7	96.1	5.7

[a] 2000 ng/mL.

tions during single and multiple dosing regimens to both extensive and poor metabolizers. As a result, a large linear dynamic range for the bioanalytical method was required. The target calibration range was 5000 times the LLOQ for each analyte, resulting in ULOQs of 50 ng/mL for DEX/DET and 5000 ng/mL for GG, given the established LLOQs of 0.010/0.010/1.0 ng/mL for these compounds. To achieve acceptable linearity throughout this range, a weighted curve fit and multiple injection volumes (Section 3.4) were utilized. Sample dilution with blank plasma was also required for those samples that exceeded the upper end of this calibration range.

Residuals from calibration curves produced during the five validation batches are displayed in Tables 2–4 for DEX, DET, and GG, respectively. The average correlation coefficients for these calibration curves were 0.9996, 0.9994, and 0.9992 for DEX, DET, and GG. These data confirm very good linearity and accuracy of the calibration standards across the entire calibration range of all analytes.

Because lower maximum analyte concentrations were expected in studies 4–6 and the high level QC samples and standards exhibited some negative bias following validation, the highest calibration standard and QC sample were reduced by a factor of two for studies 4–6. The cause of the observed negative bias, especially with samples containing high levels of DEX and GG, was not definitively determined; however, in the course of normal preventative maintenance, a new multiplier was installed in the mass spectrometer following the completion of validation, which appeared to coincide with the lower recoveries of the higher concentration standards and QC samples.

To fit the calibration data, a $1/x^2$ linear function was utilized within the MacQuan software program. The weighted fit was essential for characterizing the large dynamic range. However, this version of software did not have a $1/x^2$ quadratic function, which generally provides a better fit for high concentration standards that are most susceptible to negative deviation from linearity. Use of this weighted quadratic function, available with newer software packages such as Analyst marketed by AB/MDS Sciex, would likely result in consistently better accuracies for high level calibration standards and QC samples. In addition, newer triple quadrupole mass spectrometers deliver wider linear dynamic ranges than the API 3000. However, even with newer mass spectrometers, the use of both optimal curve fitting and variable injection volumes are useful techniques for extending calibration ranges, when needed.

The large linear range reduced the need for sample dilution and re-preparation; however, dilution was still occasionally necessary for samples containing unknown concentrations above the ULOQs. During validation, the analyses of triplicate samples spiked at 250/250/25,000 ng/mL DEX/DET/GG and diluted 1:10 with blank plasma resulted in average percent accuracies (percent CV in parentheses) of 102.9 (1.8), 105.5 (6.7), and 102.3 (1.7) for DEX/DET/GG, respectively. These data confirmed that when a 20 μL aliquot of plasma is sampled and diluted with 180 μL of blank plasma, accurate and precise quantitative measurements are obtained.

3.4. Injection volume and signal saturation

In addition to utilizing a weighted calibration curve fit, the use of multiple injection volumes was investigated for extending the linear range of the assay. At approximately 1000 times the LLOQ with an injection volume of 50 μL, signal saturation was approached, most noticeably for DEX and GG, resulting in a negative deviation from the other points on the calibration curve. The peak intensity at this calibration level was approximately 1000-fold higher than the LLOQ peak intensity, which produced a signal-to-noise ratio of approximately ten. By reducing the injection volume of the highest standards, QCs, and study samples, the corresponding peak intensity was reduced and the linear range was extended by an effective factor of five.

To optimize the use of variable injection volumes, spiked calibration standards were analyzed to determine which volumes provided adequate analyte and internal standard signal-to-noise ratios, did not result in analyte signal saturation, and produced acceptable accuracy and precision. The injection volumes of 2, 5, 10, and 50 μL were used for analysis of each calibration standard containing all three analytes. The resulting data for each analyte were plotted on a calibration curve to determine which injection volumes produced acceptable calibration data based on calculated accuracies. Generally, a 50 μL injection volume could be used for calibration standards in the lowest three orders of magnitude and 5 μL typically sufficed for the upper portions of the curves.

Using the selected injection volumes and the corresponding peak area ratios to establish calibration curves for each analyte, a spiked standard at 1/1/100 ng/mL was then analyzed in triplicate with injection volumes of 2, 5, 10, 20, and 50 μL. For these analyses, the respective accuracies (percent CV) for DEX were 96.4 (8.3), 97.3 (4.0), 96.1 (2.7), 101.3 (1.4), and 99.0 (1.1)%. For DET, the accuracies were 110.0 (7.9), 93.3 (8.4), 103.2 (13.9), 105.2 (3.7), and 103.0 (7.3)%, while GG provided corresponding results of 98.6 (12.1), 92.4 (3.1), 99.0 (4.8), 99.2 (2.1), and 100.3 (1.7)%. Characteristics of this assay that contributed to consistent peak area ratios when varying injection volume include: reproducible retention times, no significant ionization suppression (Section 3.8), and the use of stable-labeled internal standards.

The capability of extending the calibration range 5-fold by reducing the injection volume greatly decreased the number of unknown samples requiring re-preparation with the over-range procedure. Adjusting the injection volume was shown to be a flexible and rapid post preparation process to extend the linear range. Analyte signals that were too intense with the 50 μL injection were simply reinjected using a lower volume. For samples where analyte levels were expected to be on the lower end of the calibration range for some analyte(s) and relatively higher for others, two different injection volumes were utilized in an attempt to obtain data for all analytes during the initial batch run. However, in cases where samples contained at least one analyte with a measured peak area ratio exceeding the upper end of the calibration range, re-preparation was performed by 1:10 dilution in blank plasma prior to analysis.

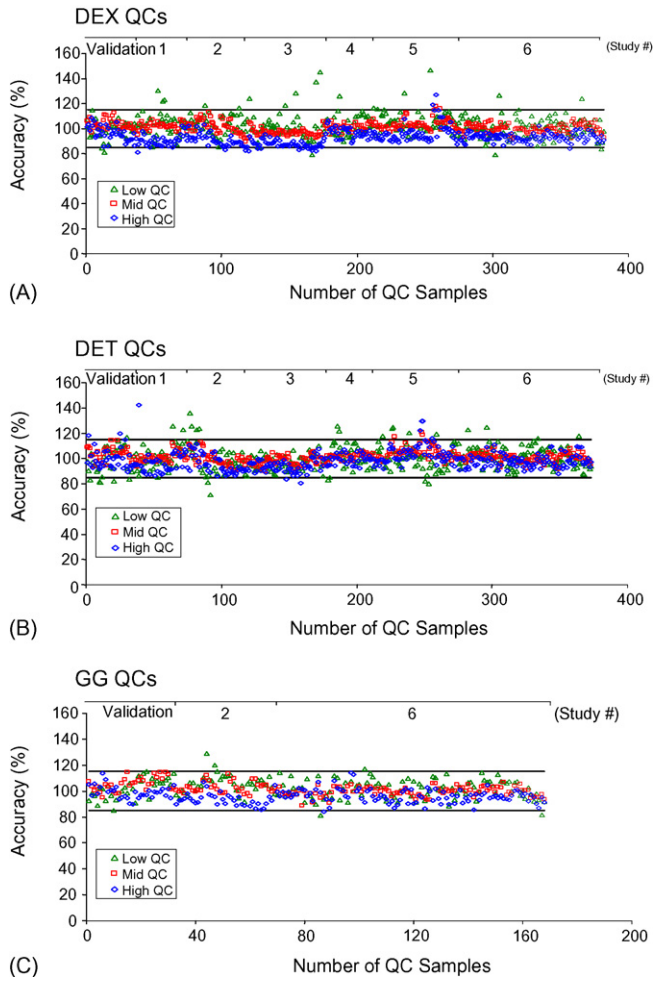

Fig. 3. Accuracies of QC samples analyzed during validation and clinical sample analysis for (A) DEX, (B) DET, and (C) GG.

3.5. Accuracy and precision

The accuracy and precision of the method, as indicated by the results of QC sample analyses, were determined during validation and throughout analysis of all clinical study batches. QC data summaries for average accuracy and precision (expressed as percent CV) for each of these studies are found in Tables 5–7 for DEX, DET, and GG, respectively. Summary data at all QC levels easily met success criteria of ±15% for average accuracy (±20% at the LLOQ) with CVs of ≤15% (≤20% at the LLOQ). Control charts of the QC accuracies determined over this period are plotted in Fig. 3.

A total of 2859 QC samples were analyzed during validation and clinical sample analysis. All QC accuracies within the range of 50–150% of target are included in Tables 5–7 and plotted in Fig. 3. However, eight QC samples (0.3%) fell outside the ±50% range. Six of these outliers were low level DEX QC samples, one was a low level DET QC, and one an LLOQ DET QC. Throughout method validation and application, a total of 3.8% of the 2859 QCs fell outside of the target specifications, meaning that 96.2% were within the acceptable range. While accuracy and precision could potentially be improved, when comparing this level of performance with FDA guidance criteria for acceptable batches allowing 33% of QCs to be outside of ±15%, the overall performance of less than 4% of QCs outside of the target range is excellent.

3.6. Autosampler carry-over

Injection of a diluent blank immediately following analysis of the highest calibration standard resulted in no observed response for DET or GG. DEX exhibited a response that was 0.15% of the peak area produced by injection of the highest calibration standard. For the second diluent injection, the level decreased to 0.06% and there was no measurable response from the third diluent injection. Due to the carry-over of DEX, diluent blanks were routinely injected immediately following the analysis of either the high calibration standard or a high QC sample, and immediately preceding the analysis of plasma blanks or predose study samples. Also, study samples from a given subject were analyzed in sequence based on collection times to minimize analyte concentration differences in consecutively injected samples.

3.7. Batch size and ruggedness

The analysis conditions and target batch size were designed to deliver a level of throughput that was high enough to meet project timing, but also maintain a relatively straightforward and reliable method. Samples were received from multiple clinical sites and each shipment required rapid processing to generate plasma concentration data. However, the receipt of clinical samples occurred over a period of approximately 6 months. During some weeks, as many as several hundred samples were received while during some weeks there were no samples received. Semi-automated preparation of four-plate batches with serial LC–MS/MS analysis provided a good balance between throughput and simplicity/reliability. With each four-plate batch, approximately 300 study samples were analyzed. Batches of this size were routinely prepared by two analysts in 4 h and processed on a single LC–MS/MS system with an analysis time of approximately 25 h.

The ability to analyze batches consisting of four 96-well plates was originally established during validation. In this ruggedness evaluation, average percent accuracies for DEX (percent CV in parentheses, $n = 18$) were 99.7 (6.8), 101.3 (3.3), and 100.9 (3.2) at the low, mid, and high QC levels. For DET, the accuracies were 97.2 (8.3), 103.1 (4.4), and 103.9 (7.0) for low, mid, and high level QC samples, while for GG the results for average percent accuracy were 99.0 (6.1), 101.0 (3.2), and 102.9 (4.7). Mass spectrometer sensitivity remained essentially constant throughout and there was no observed increase in HPLC column back pressure. For batches containing study samples, QCs were typically analyzed at low, mid, and high levels at the beginning, middle, and end of each plate, except on the first plate where the QC samples were run after the calibration standards and diluent blanks. Most of the QC accuracy and precision data displayed in Tables 5–7 and in Fig. 3 were collected during analysis of batches consisting of four plates.

To achieve a level of ruggedness required for consistently reliable analysis of batches comprised of four 96-well plates, several important parameters were carefully optimized in the LLE procedure. Even after centrifugation, removal and usage of the ether layer near the plasma interface was shown to be detrimental to assay ruggedness and to increase ionization suppression. By removing only the upper one-half of the ether layer, very clean extracts were produced that resulted in very little ionization suppression (Section 3.8) and a rugged analysis method. A back extraction step was not used, but rather the ethyl ether was evaporated and the extract was reconstituted in 1% formic acid. These conditions increased sample throughput, allowed the simultaneous quantification of all three analytes, and proved to be very rugged and reliable, with thousands of plasma extracts typically injected on a single HPLC column.

The capability to analyze, notebook, and review up to three batches per week provided a potential throughput of 900 clinical samples per week with one robotic workstation for sample preparation and one LC–MS/MS instrument. Methods for higher rates of bioanalytical sample analysis with MS/MS detection have been reported in the literature such as high-flow supercritical fluid chromatography [38,39], staggered parallel injections [40,41], and parallel analysis with a MUX interface [42,43]. These options were considered because of the demonstrated increased bioanalytical throughput; however, these approaches also increase complexity which creates additional opportunities for issues with ruggedness and reliability. When considering the rate of sample receipt and the requirements for turn around, the established throughput potential of 900 clinical sample analyses per week was sufficient for this application.

3.8. Extraction efficiency and ionization suppression

The analyte and internal standard extraction efficiencies from human plasma are displayed in Table 8. These efficiencies include losses due to recovering only 300 μL of the total 600 μL ethyl ether extraction volume. Method optimization revealed that attempting removal of the entire ether layer introduced contaminants that resulted in increasing HPLC back pressure and degradation of analyte peak shapes after a few hundred injections. However, with stable-labeled internal standards to compensate for analyte losses, the accuracy and precision remained excellent and sensitivity was not significantly compromised.

Table 8
Extraction efficiencies of DEX, DEX ISTD, DET, DET ISTD, GG, and GG ISTD from human plasma

Compound	Extraction efficiency (%)		
	Low QC	Mid QC	High QC
DEX	59.5	51.6	41.4
DEX ISTD	53.9	51.1	40.4
DET	52.1	52.7	38.0
DET ISTD	49.8	50.8	41.4
GG	18.0	19.8	17.8
GG ISTD	20.3	19.7	15.4

To evaluate additional potential sources of signal loss, such as ionization suppression, the MS/MS responses from the analysis of spiked, blank plasma subjected to the preparation procedure were compared with the analogous responses obtained from neat solutions. In all cases, the peak areas measured from the plasma analysis compared to those obtained from the neat determinations were essentially equal on a percentage basis (all within 4%) to the results obtained for the extraction efficiency investigation (Table 8). Since the current experiment measures extraction efficiency plus all additional sources of signal loss, these results indicate that after accounting for losses due to recovery from plasma, there are no other significant sources of response loss, including ionization suppression. These data also provide additional evidence that the automated preparation conditions produce very clean sample extracts.

3.9. Stability

Analyte stability was investigated for a variety of conditions that were utilized for handling and storage of both standards and samples. Stability was confirmed if after exposure to a given condition for a period of time, average measured analyte concentrations were ±15% of their respective spiked concentrations. Results of the stability studies are summarized in Table 9. These studies demonstrated that all three analytes are stable in spiking solutions of water–methanol (75:25, v/v) with 0.1% sodium chloride when stored in Sarstedt polypropylene cryovials at 4 °C for 34 days. Analytes were also determined to be stable in sodium heparin tubes containing the fresh whole blood when stored at room temperature for up to 2 h, allowing an interval as long as 2 h between collection and removal of the plasma.

Analyte stability in plasma was studied extensively as this is the key matrix for PK studies. Long-term stability of plasma samples stored frozen at −70 °C for up to 1 year was demonstrated. Freeze/thaw cycles were also investigated to assure that no analyte losses occurred during the sample freezing and thawing that were required for analysis and possible sample retests. All three analytes were shown to be stable for as many as three freeze/thaw cycles with temperature changes from ambient to −70 °C followed by a return to room temperature. Short-term storage of plasma at room temperature on the bench top was evaluated to simulate worst case exposure to room temperature after thawing and during possible delays in the sampling process prior to refreezing. Analyte stability was demonstrated for up to 6 h under these conditions. For prepared samples, stability was established to insure that if instrument or operator time were not immediately available, prepared samples could be stored refrigerated for up to 12 days prior to analysis. As indicated in Table 9, for each condition, all three analytes were determined to be stable over the investigated time periods.

3.10. Pharmacokinetic results

Mean plasma concentration versus time profiles for DEX, DET, and GG following oral administration of two formulations are displayed in Figs. 4 and 5 for extensive and poor metabolizers, respectively. The primary differences observed

Table 9
Stability of DEX, DET, and GG under various storage conditions

Storage condition	Dextromethorphan				Dextrorphan				Guaifenesin			
	Low level		High level		Low level		High level		Low level		High level	
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
Spiking solution (34 days, 4 °C)	100.6	5.7	104.4	0.9	110.6	6.1	112.9	4.3	109.3	6.0	110.4	5.9
Blood (1 h, 22 °C)	102.0	5.7	100.1	2.8	110.7	2.0	111.7	1.9	98.7	5.7	98.6	4.3
Blood (2 h, 22 °C)	95.8	7.7	98.9	1.8	102.7	3.7	109.3	7.3	94.5	1.3	98.4	2.3
Plasma (2 h, 22 °C)	107.1	10.6	100.4	0.8	92.8	8.1	101.5	9.6	95.7	0.6	98.8	5.6
Plasma (6 h, 22 °C)	100.9	6.6	98.2	1.8	93.7	2.9	106.4	9.4	94.1	3.3	102.4	2.9
Plasma (3× F/T, −70 °C)	101.1	5.5	98.6	2.3	97.9	6.6	99.9	4.1	99.3	6.6	104.6	6.1
Plasma (55 days, −70 °C)	108.1	7.8	97.4	1.6	102.1	6.6	98.4	3.6	110.3	4.7	93.0	2.6
Plasma (6 months, −70 °C)	106.2	3.8	95.4	2.3	106.5	2.9	101.1	4.0	103.6	1.2	94.7	3.3
Plasma (1 year, −70 °C)	95.8	6.5	88.2	4.3	95.3	3.9	102.3	3.4	98.1	4.3	93.0	4.9
Extract (12 days, 4 °C)	92.4	8.7	93.8	7.7	102.5	7.1	100.0	5.3	100.0	5.2	94.2	3.8

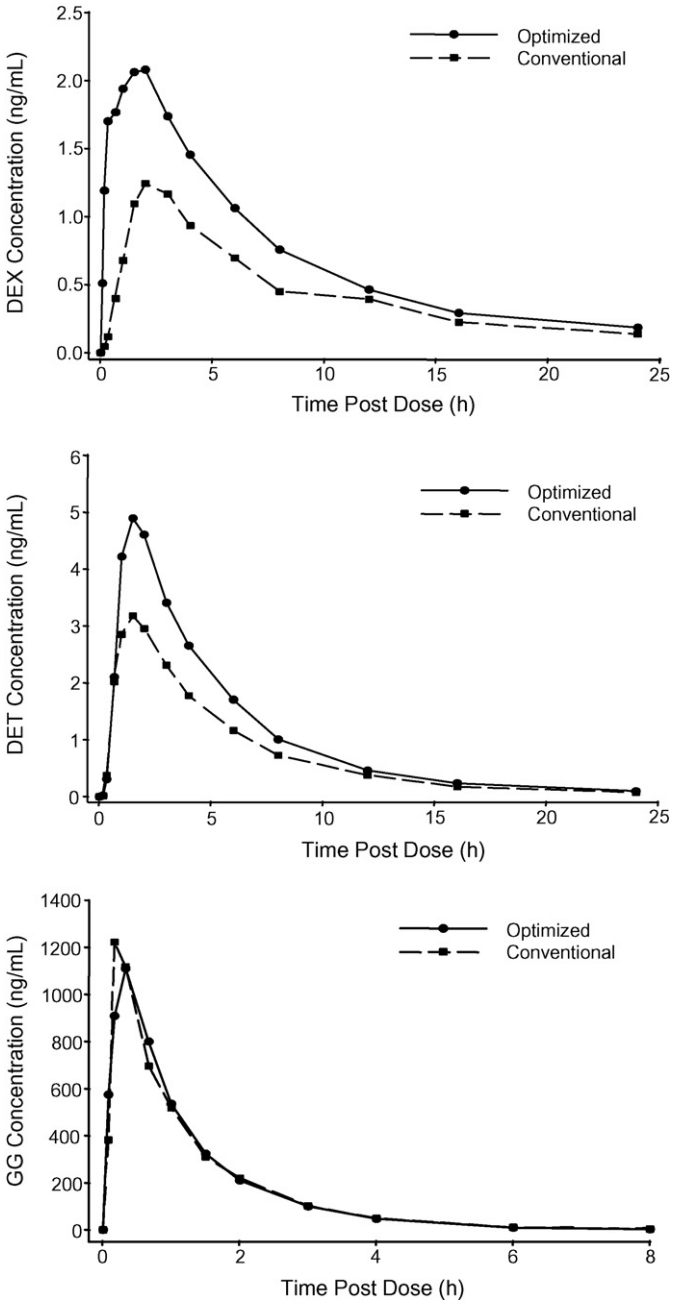

Fig. 4. Mean plasma concentrations of DEX, DET, and GG following administration of either an optimized or conventional formulation containing DEX and GG to 20 extensive metabolizers.

after administration of the conventional and optimized formulations are found in the DEX plasma concentration–time profiles of the extensive metabolizers (Fig. 4). After administering the optimized formulation to extensive metabolizers, DEX plasma concentrations were approximately 120-fold higher at 5 min and the area under the DEX plasma concentration curve over the first hour, $AUC_{0-1\,h}$, was approximately 5-fold higher. In addition, a 2-fold increase in C_{max} and AUC along with an earlier t_{max} were observed following administration of the optimized formulation. In poor metabolizers, DEX plasma concentration–time profiles were generally similar following oral administration of the conventional and optimized formulations (Fig. 5). However,

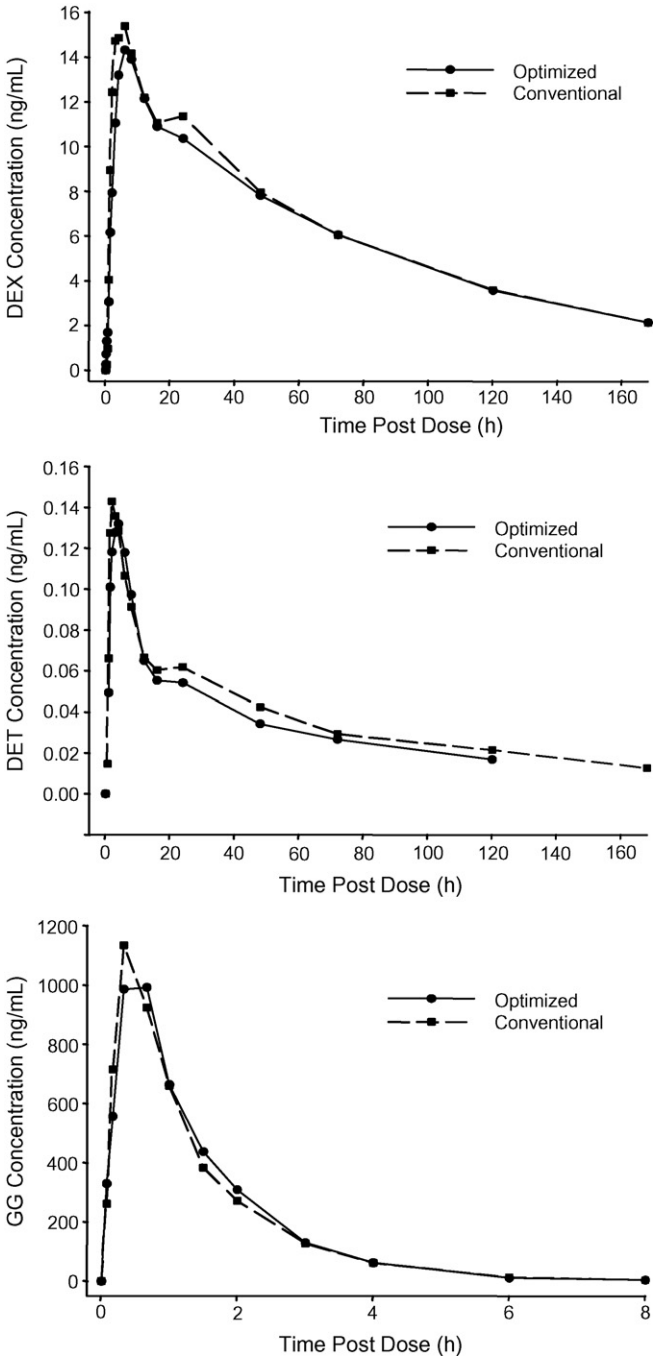

Fig. 5. Mean plasma concentrations of DEX, DET, and GG following administration of either an optimized or conventional formulation containing DEX and GG to 8 poor metabolizers.

a noteworthy difference was observed during the first 20 min following administration, when DEX plasma concentrations were 5–10-fold higher for the optimized formulation. No other formulation related differences were observed for any other pharmacokinetic parameter.

For dextrorphan, an increase in C_{max} and AUC (~1.5-fold) were observed following administration of the optimized formulation to extensive metabolizers with no differences observed in poor metabolizers. For guaifenesin, no differences in plasma concentrations, C_{max}, or AUC were noted in either extensive or poor metabolizers. The pharmacokinetic data for DEX and DET provide the expectation of more rapid and effective cough relief with the optimized product. These expectations are supported with published study results for formulations containing DEX as the only active [44]. With the referenced study, a more rapid and efficient absorption of DEX from an optimized formulation was observed when compared to a conventional product. In addition, efficacy data confirmed the direct relationship between cough reductions and measured plasma concentrations of DEX.

4. Conclusions

The combination of semi-automated LLE with gradient elution LC–MS/MS provides a very reliable and rugged methodology for the ultratrace, high-throughput analysis of plasma samples to quantify multiple target analytes with a range of compound functionalities. The use of stable-labeled internal standards for each analyte facilitates accurate and precise quantification over a wide dynamic range with a single set of extraction and LC conditions by compensating for losses when using conditions that are not optimal for each analyte. Also, stable-labeled internal standards allow the utilization of only one-half of the organic layer from the extraction. This produces very clean extracts and provides excellent ruggedness of the LC–MS/MS analysis with virtually no ionization suppression.

The method for the simultaneous quantification of DEX, DET, and GG was successfully applied to the analysis of clinical pharmacokinetic samples collected from six studies that yielded more than 6200 plasma samples. Two or three analytes were determined in each sample resulting in more than 15,000 unknown concentration measurements in plasma. During that period, the ruggedness and reliability of the method was well established by analysis of 42 batches including those designed for validation, clinical samples, and repeat analyses. The typical batch consisted of four 96-well plates that were prepared by two analysts in an afternoon and analyzed in approximately 25 h of LC–MS/MS instrument time. Assay accuracy and precision were monitored through recovery of QC samples and were shown to be excellent with typical average accuracies within a few percent of target and CVs of less than 8% for most studies. Examples of PK results demonstrate the utility of this methodology, and bioanalytical measurements in general, for designing superior products and confirming their performance by measuring drug delivery and correlating with biological efficacy.

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