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# Simultaneous determination of tolbutamide, omeprazole, midazolam and dextromethorphan in human plasma by LC–MS/MS—A high throughput approach to evaluate drug–drug interactions

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ABSTRACT

Drug–drug interactions involving cytochrome P450 (CYP450s) are an important factor for evaluation of a new chemical entity (NCE) in drug development. To evaluate the potential inhibitory effects of a NCE on the pharmacokinetics of a cocktail of representative probes of CYP enzymes (midazolam for CYP3A4, tolbutamide for CYP2C9, omeprazole for CYP2C19 and dextromethorphan for CYP2D6) and the safety and tolerability of the NCE in the presence of probe substrates, a high throughput liquid chromatography/tandem mass spectrometry (LC–MS/MS) method was developed and validated for the simultaneous determination of tolbutamide, omeprazole, midazolam and dextromethorphan in human plasma using tolbutamide-$d_9$, midazolam-$d_4$, (±)-omeprazole-$d_3$, and dextromethorphan-$d_3$ as the internal standards (ISs). Human plasma samples of 50 μL were extracted by a simple protein-precipitation procedure and analyzed using a high performance liquid chromatography electrospray tandem mass spectrometer system. Reversed-phase HPLC separation was achieved with a Hypersil GOLD AQ column (50 mm × 4.6 mm, 5 μm). MS/MS detection was set at mass transitions of 271 → 172 *m/z* for tolbutamide, 346 → 198 *m/z* for omeprazole, 326 → 291 *m/z* for midazolam, 272 → 171 *m/z* for dextromethorphan, 280 → 172 *m/z* for tolbutamide-$d_9$ (IS), 349 → 198 *m/z* for (±)-omeprazole-$d_3$ (IS), 330 → 295 *m/z* for midazolam-$d_4$ (IS), and 275 → 171 *m/z* for dextromethorphan-$d_3$ (IS) in positive mode. The high throughput LC–MS/MS method was validated for accuracy, precision, sensitivity, stability, recovery, matrix effects, and calibration range. Acceptable intra-run and inter-run assay precision (<10%) and accuracy (<10%) were achieved over a linear range of 50–50,000 ng/mL for tolbutamide, 1–1000 ng/mL for omeprazole, 0.1–100 ng/mL for midazolam and 0.05–50 ng/mL for dextromethorphan in human plasma. Method robustness was demonstrated by the 100% pass rate of 24 incurred sample analysis runs and all of the 50 clinical study samples used for incurred sample reproducibility (ISR) test having met the acceptance criterion (%Diff within 20%). The overall ISR results for all compounds showed that over 95% of the samples had a %Diff of less than 10%. The method is simple, rapid and rugged, and has been applied successfully to sample analysis in support of a drug–drug interaction study.

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## 1. Introduction

Identifying drug–drug interaction (DDI) potential early in drug discovery and development is important because drug–drug interactions can cause life threatening changes in drug levels, which is a leading cause of death in the US [1,2]. Identifying potential DDI expedites the decision to eliminate that compound from consideration, thus lowering the cost of drug discovery and development. The current DDI draft guidance calls for a clinical DDI study if more than 25% of the drug clearance is from a specific pathway (Food and Drug Administration Drug–Drug Interaction Draft Guidance, 2006, http://www.fda.gov/cder/guidance/6695dft.htm) and accepts that simultaneous administration of a mixture of substrates of CYP enzymes in one study (i.e., a "cocktail approach") in human volunteers to evaluate a drug's interaction potential, provided that the substrates are specific for individual CYP; there are no interactions among these substrates; and the study is conducted in a sufficient number of subjects. Numerous examples are present

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describing well-tolerated cocktail combinations that allow for the simultaneous investigation of drug–drug interactions for specific CYP450s [3–8]. To evaluate the potential inhibitory effects of a New Chemical Entity (NCE) on the pharmacokinetics of a cocktail of representative probes of CYP enzymes and the safety and tolerability of the NCE in the presence of probe substrates, midazolam, tolbutamide, omeprazole, and dextromethorphan have been selected as probe substrates for CYP3A4, CYP2C9, CYP2C19 and CYP2D6, respectively. This is because none of these four substrates are competitive inhibitors or inducers of CYP metabolism, which makes them widely used probes in the drug–drug interaction research. To evaluate the pharamacokinetic parameters of these four probe substrates, a reliable and high throughput analytical method is needed to measure the concentrations of these four compounds in clinical study samples.

Due to its speed, sensitivity, and selectivity, liquid chromatography/tandem mass spectrometry (LC–MS/MS) has become the method of choice for analyzing drug candidates in biofluids [9–11]. In vitro metabolic interaction screening assay for determination of marker substrates of CYP450s using N-in-1 approach and LC–MS/MS have been reported earlier [12–15]. However, to date, no published reports have dealt with the simultaneous quantitation of tolbutamide, omeprazole, midazolam, and dextromethorphan in human plasma for in vivo drug–drug interaction study. In this paper, we present a sensitive, specific, and rapid 4-in-1 LC–MS/MS method for the simultaneously analyzing of these four marker substrates of CYP450s in human plasma. The purpose of this work is to develop an LC–MS/MS method that can be used to simultaneously quantitate all these four compounds in a single injection. This method should also have a fast run time so that analysis of the samples can be accomplished in a timely fashion to support the clinical study. The reproducibility and ruggedness of the method need to be demonstrated by incurred plasma sample reanalysis.

## 2. Experimental

### 2.1. Chemicals and reagents

Tolbutamide (purity >99%) and omeprazole (purity 99.9%) were purchased from USP (Rockville, MY, USA). Midazolam (purity 98%) and dextromethorphan-$d_3$ (purity 98%) and dextromethorphan (purity 99.9%) were purchased from Toronto Research Chemicals (North York, ON, Canada). Tolbutamide-$d_9$ and (±)-omeprazole-$d_3$ were purchased from CDN Isotopes, Inc. (Pointe-Claire, Quebec, Canada). Midazolam-$d_4$ maleate was purchased from Cerilliant (Round Rock, TX, USA). The chemical structures of tolbutamide (TOL), omeprazole (OME), midazolam (MID), dextromethorphan (DEX) and their internal standards are shown in Fig. 1. Purified water (18.2 Ω cm) was processed with ELGA Pure Lab Classic water polisher and deionizing unit (Marlow, UK). Acetonitrile and methanol were HPLC grade and from Mallinckrodt (Phillipsburg, NJ, USA). Ammonium formate, ammonium carbonate ($Na_2CO_3$), sodium hydroxide (50%) and sodium bicarbonate are GR grade from Sigma (St. Louis, MO, USA). Formic acid was GR grade from Spectrum (Gardena, CA, USA). Blank human plasma with potassium EDTA ($K_2$EDTA) as anticoagulant was from Bioreclamation Inc. (Hicksville, NY, USA) and was stored in a freezer at −20 °C.

### 2.2. Calibration standards and quality control samples

Standards and QC samples were made from two separate stock solutions 1 mg/mL of each analyte as free base in methanol/water (50:50, v/v) except for omeprazole in methanol/water/50% NaOH with ~0.36% of $Na_2CO_3$ (20/50/0.2, v/v/v). For the validation work, these two stock standard solutions must agree to within 5% with the LC–MS/MS response. The stock solutions were stored in polypropylene tubes with screw caps and were stable for at least 60 days when kept in a refrigerator at 2–8 °C and protected from light and for at least 6 h when kept at room temperature (22 °C). The working solutions of midazolam and dextromethorphan were prepared in a solution mixture of methanol and water (50:50, v/v) at concentrations of 100 μg/mL and 50 μg/mL, respectively. These working solutions were stored in a refrigerator at 2–8 °C and were used to prepare calibration standards and QC samples within 24 h after preparation. Pooled (4-in-1) calibration standards containing the mixture of each analyte at concentrations of 50/1/0.1/0.05, 100/2/0.2/0.1, 500/10/1/0.5, 2500/50/5/2.5, 5000/100/10/5, 20,000/400/40/20, 40,000/800/80/40, and 50,000/1000/100/50 ng/mL of TOL/OME/MID/DEX were prepared in blank plasma pool made by combining six lots of blank plasma. QC samples at levels of 150/3/0.3/0.15, 4000/80/8/4, and 39,000/780/78/39 ng/mL (TOL/OME/MID/DEX) were prepared for the determination of intraday and interday accuracy and precision.

Over-the-curve QC samples were prepared at 100,000/2000/200/100 ng/mL and lower limit of quantitation (LLOQ) QC samples were prepared at 50/1/0.1/0.05 ng/mL. The volumes of the spiking solutions were always kept below 5% of the plasma volumes. All standards and QC samples were aliquoted (0.20 mL) into pre-labeled 2-mL polypropylene vials and stored frozen at −20 °C. Stock solution of internal standard (IS, 1 mg/mL) was prepared in methanol/water (50:50, v/v) except for omeprazole-$d_3$ in methanol/water/50% NaOH methanol/water/50% NaOH with ~0.36% of $Na_2CO_3$ (20/50/0.2, v/v/v) and 10/1/2/2.5 (TOL/OME/MID/DEX) ng/mL of IS working solution was prepared in methanol.

### 2.3. LC–MS/MS methods

LC–MS/MS analyses were performed using a Shimadzu HPLC VP system (Kyoto, Japan) coupled to a PE Sciex API 4000 tandem mass spectrometer with positive Turbo Ionspray (Concord, ON, Canada). The analytical column was a Hypersil GOLD AQ, 50 mm × 4.6 mm, 5 μm (Waltham, MA, USA) and was kept at ambient temperature. The mobile phase consisted of 1:1 acetonitrile/methanol:water (50:50) with 10 mM ammonium formate and 0.2% formic acid at an isocratic flow rate of 0.8 mL/min. The sample injection volume was 50 μL and run time was 4 min. The injector wash solvent was 0.2% formic acid in 1:1 methanol/water. Autosampler carry-over was determined by injecting the highest calibration standard followed by an extracted blank sample. No carry-over was observed, as indicated by the lack of peaks corresponding to analytes or internal standards in the blank sample. The background noise in the blank sample was also not elevated.

The sensitivity of the multiple reaction monitoring (MRM) was optimized by testing with an infusion of 0.1 μg/mL each analyte and internal standard in mobile phase. The Ionspray needle was maintained at 5.5 kV. The turbo gas temperature was 650 °C and the auxiliary gas flow setting was 70. Nebulizing gas, curtain gas, and collision gas flows were at instrument settings of 80, 24, and 8, respectively. The declustering potentials (DP) were 66 V for tolbutamide, 95 V dextromethorphan, 48 V for omeprazole and 90 V for midazolam. The potential (EP) were 15 V for tolbutamide, 9.5 V dextromethorphan, 4.8 V for omeprazole and 14 V for midazolam. The mass spectrometer was operated in MRM mode with collision energy (CE) of 18.5 eV for tolbutamide, 54 V dextromethorphan, 32 V for omeprazole and 40 V for midazolam. The collision cell exit potential (CXP) were 8.5 V for tolbutamide, 10 V for omeprazole, 15 V for midazolam and 7.6 V for dextromethorphan. The transitions (precursor to product) monitored were $m/z$ 271 → 172 for tolbutamide, $m/z$ 346 → 198 for omeprazole, $m/z$ 326 → 291 for midazolam, $m/z$ 272 → 171 for dextromethorphan, $m/z$ 280 → 172

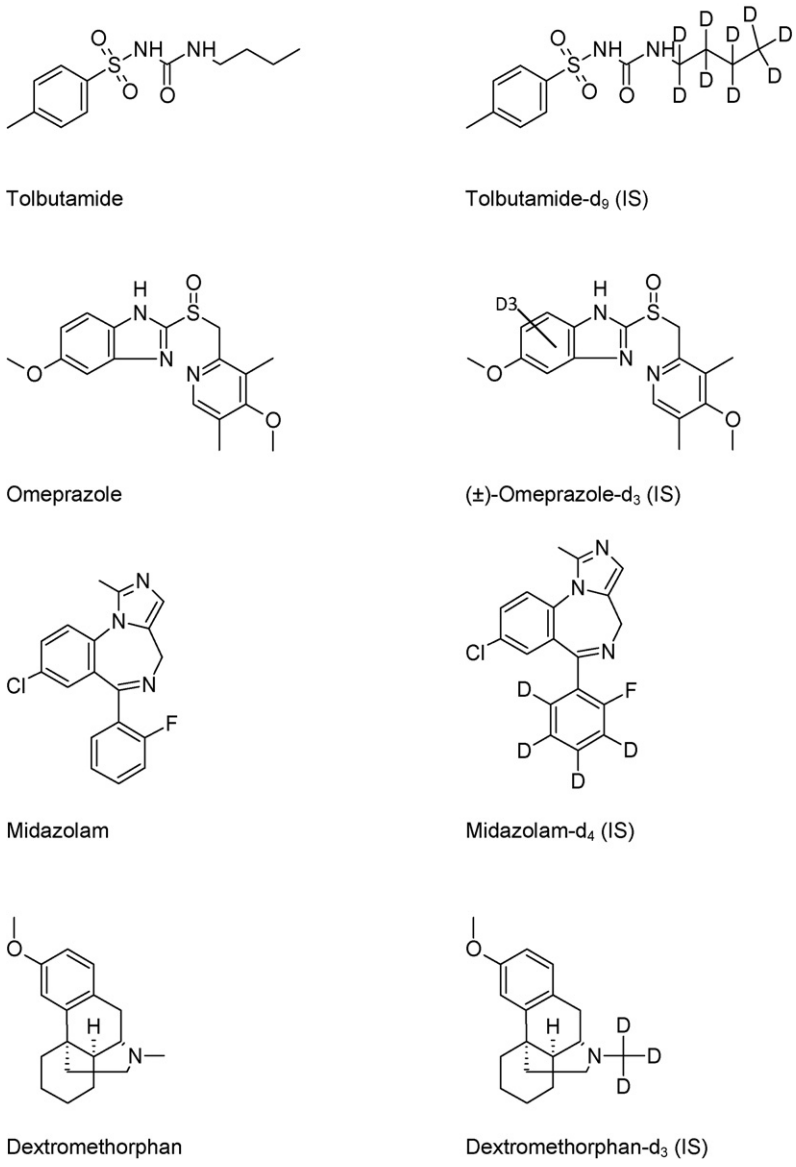


**Fig. 1.** Chemical structures of tolbutamide, omeprazole, midazolam, dextromethorphan, and the internal standards (ISs).

for tolbutamide-$d_9$ (IS), $m/z$ 349 → 198 for (±)-omeprazole-$d_3$ (IS), $m/z$ 330 → 295 for midazolam-$d_4$ (IS), and $m/z$ 275 → 171 for dextromethorphan-$d_3$ (IS) in positive mode. The dwell time was 50 ms for omeprazole, midazolam and the internal standards and 100 ms for tolbutamide and 80 ms for dextromethorphan. Both Q1 and Q3 quadrupoles were maintained at unit resolution.

### 2.4. DDI study information

This is an open-label, single-center, drug–drug interaction study of the effect of the NCE on midazolam, tolbutamide, omeprazole, and dextromethorphan in healthy male subjects. The study was reviewed and approved by the local Investigational Review Boards. Up to sixteen (16) healthy male subjects are enrolled. Single doses of midazolam (oral, single 2.5 mg dose), tolbutamide (oral, single 250 mg dose), omeprazole (oral, single 20 mg dose), and dextromethorphan (oral, single 30 mg dose) were administered in combination on four occasions: before the NCE dosing, together with a single dose of NCE at the end of 1 week of multiple oral dosing of NCE, and 1 week after cessation of treatment with NCE.

The cocktail approach has been, in general, proposed as a screening tool for potential in vivo drug–drug interactions. Negative results from a cocktail study can eliminate the need for further evaluation of particular CYP enzymes. However, positive results can indicate the need for further in vivo evaluation to provide quantitative exposure changes (such as AUC and $C_{max}$). The purpose of this study is the clinical assessment of the inhibitory potential of the drug candidate on CYP3A4, CYP2C9, CYP2C19, and CYP2D6 after single oral doses and multiple oral doses of the drug candidate in healthy adult subjects. Single doses of these four probes were administered in combination on four occasions: before the drug candidate dosing (Day 1), together with a single dose of drug candidate (Day 5), at the end of 1 week of multiple oral dosing of drug candidate (Day 12), and 1 week after cessation of treatment with the drug candidate (Day 19). Plasma samples were analyzed to determine concentrations of midazolam, tolbutamide, omeprazole, and dextromethorphan. $C_{max}$ and AUC values were selected for the analysis of a potential interaction, with the Day 1 values serving as the reference range for each probe substrate. Measurement of metabolites of the probe substrates is only required when a significant interaction was observed.

Blood samples for the measurement of probe substrates were collected at 0 (pre-dose), 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 18, 24, 36 and 48 h post-dose.

### 2.5. Sample preparation

All samples, quality control samples, and standards with a sample volume of 50 μL spiked with 500 μL of IS working solution (10/1/2/2.5 ng/mL of TOL/OME/MID/DEX in methanol) except for blanks to which 500 μL of methanol was added were vortex-mixed thoroughly followed by centrifugation at 3000 rpm for 10 min using a Beckman Coulter GS-6R centrifuge (Fullerton, CA, USA). 150 μL of supernatant was transferred into 1 mL HPLC autosampler vial. After add 150 μL of 10 mM $NH_4CO_3$ solution to each autosampler vial and centrifugation for 5 min at 3000 rpm, an aliquot of 50 μL of each sample was then injected onto the LC–MS/MS system for analysis.

### 2.6. Validation of the LC–MS/MS method

The method was validated for accuracy, precision, sensitivity, specificity, calibration curve range, and reproducibility according to the FDA guideline for bioanalytical methods validation [16] over a concentration range of 50/1/0.1/0.05 to 50,000/1000/100/50 ng/mL of TOL/OME/MID/DEX using eight calibration standards, each containing the four analytes of interest, and six replicates of QC samples at each concentration level in three separate runs. The method sensitivity with the target lower limit of quantitation (LLOQ) of 50/1/0.1/0.05 ng/mL for TOL/OME/MID/DEX in human plasma was validated. One calibration curve contained the over-the-curve QC samples (100,000/2000/200/100 ng/mL of TOL/OME/MID/DEX), which were diluted 20-fold with control blank plasma prior to analysis. Each batch also contained other test samples such as processing and storage stability samples. Calibration standards, QC samples, and other test samples were randomized throughout the run. A blank sample fortified with the internal standards was always included in each batch. An extracted blank sample was always placed after the ULOQ standard (upper limit of quantitation) to determine carry-over of the LC–MS/MS system. The method specificity was evaluated by screening six lots of blank plasma. The plasma samples were extracted and analyzed to confirm lack of interference and absence of significant lot-to-lot variation. In addition, interference between tolbutamide, omeprazole, midazolam, dextromethorphan, and the NCE at their respective retention times was evaluated.

Analyte stability was tested using QC samples for multiple freeze/thaw (F/T) cycles, on the bench at room temperature (short-term stability), or at −20 °C in the freezer (long-term storage). Post-preparative stability and stock solution stability were also determined. To assess the stabilities of tolbutamide, omeprazole, midazolam, and dextromethorphan in whole blood at two temperatures (ice bath, wet ice, 0–4 °C and room temperature) and three time periods (0, 30, and 120 min) were evaluated. The overall evaluation of extraction recovery and matrix effect of four analytes was calculated by comparing the peak areas of extracted plasma standards to the peak areas of neat solutions spiked at corresponding concentrations. To evaluate the incurred sample reproducibility (ISR) of the method, 50 clinical sample samples for tolbutamide, omeprazole, midazolam, and dextromethorphan were selected from the clinical study per pre-approved ISR study plan.

Retention time and peak area were determined by Analyst Data Acquisition/Processing Software (Version 1.4.2). Analyte concentrations were obtained from a calibration curve constructed by plotting the peak area ratio versus the concentration using Watson LIMS (Version 7.3). The calibration curve was calculated using the simplest available relationship (usually a linear model). The curve fitting model and weighting function selected was used for all batches evaluated during method validation.

## 3. Results and discussion

### 3.1. LC–MS/MS method development

The goal of this study is to develop and validate a simple, fast and reliable LC–MS/MS method to measure the four probes simultaneously. The unique challenge for developing and validating quantitative bioanalytical method to support DDI study is that there are several probes that can be used for each iso-enzyme and there is no industry consensus on standardizing the probe selection. The “Pittsburgh cocktail” used caffeine, chlorzoxazone, dapsone, debrisoquin and mephenytoin as probes for CYP 1A2, 2E1, 3A4, 2D6, and 2C19, respectively [17]. In the “GW Cocktail”, caffeine, diclofenac, mephenytoin, debrisoquine, chlorzoxazone, and midazolam were recommended as probes for CYP 1A2, 2C9, 2C19, 2D6, 2E1, and 3A4, respectively. Attempt was made to reach a consensus on assess drug–drug interaction studies [18] and several preferred and acceptable probes were suggested for each CYP. Caffeine, tolbutamide, mephenytoin or omeprazole, debrisoquine, and midazolam or simvastatin were recommended for CYP 1A2, 2C9, 2C19, 2D6, and 3A4, respectively. Nevertheless, new combinations of probes continued to appear in literatures. For example, a modified “Cooperstown 5 + 1 Cocktail” used caffeine (CYP1A2), dextromethorphan (CYP2D6), omeprazole (CYP2C19), and intravenous midazolam (hepatic CYP3A). Warfarin (administered with vitamin K, the pharmacodynamic effect is ablated) was used as a safe and accurate biomarker for CYP2C9 [19]. LC–MS/MS method was validated for theophulline, tolbutamide, mephenytoin, debrisoquin, and dapsone as probes for CYP 1A2, 2C9, 2C19, 2D6, and 3A4 [20]. Depending on the study design, therapeutic area of the drug candidate, potential co-administered medicine, and physician or clinic site’s preference, several combinations of cocktails can be employed and not all probes may be included in the in vivo studies if the in vitro data suggested lack of evidence for certain probes. When propiverine was investigated in healthy volunteers for its effect on CYP450 enzymes, Only CYP 3A4 (midazolam), 2C9 (tolbutamide), 2C19 (mephenytoin), and 1A2 (caffeine) were monitored. The “Cocktail” approaches and strategies in drug development have been reviewed and some potential drawback such as lack of agreed cocktail approach has been highlighted [21]. Method validation has to be conducted for each combination. Even though there are some published LC–MS/MS methods which combined with in vivo or in vitro cocktail approaches, none of them included the same four-specific probe substrates as requested in our clinic study. The choice of the probes for the other CYPs was based on the in vitro study, the literatures and the previous experiences of the investigator. Within in vitro testing, the NCE has demonstrated concentration-dependent inhibition of human CYP2D6, CYP3A4, CYP2C9, and CYP2C19. Therefore, only probe substrates for these four CYP isoforms were selected in this study and CYP 1A2 has been excluded from the current in vivo study. For all probe substances, the oral dose used in the DDI studies is always substantially lower than the therapeutical starting dose range [3]. All these four probes have been commonly used in the literatures.

In this paper, we present an LC–MS/MS for the simultaneous analysis of these four probes. In order to achieve rapid analysis, a number of columns that are amenable to high speed analysis but without sacrificing chromatographic performance were evaluated. These include sub-2 micron columns, monolithic columns and silica-based HILIC columns. It was found that the optimal combination of peak shape, tailing factor, retention time, and back pressure

were achieved using a Hypersil GOLD AQ HPLC column with the mobile phase consisted of 1:1 acetonitrile/methanol:water (50:50) with 10 mM ammonium formate and 0.2% formic acid was used for separation. Good separation of tolbutamide, omeprazole, midazolam, and dextromethorphan and the internal standards was achieved, and maintained good peak shapes with the retention times at ~2.5 min for tolbutamide, ~1.5 min for omeprazole, ~2.1 min for midazolam, and ~2.2 min for dextromethorphan. All analytes were well retained on the column and were well separated from the solvent front ($t_0$ = 0.5 min) where the matrix suppression could be the most problematic.

A simple protein precipitation method was employed. An LC–MS/MS method in conjunction with protein precipitation is well suited for simultaneous analysis of analytes possessing diverse polarities. Omeprazole is quite polar while tolbutamide is relative hydrophobic. One concern for protein precipitation methods is that this type of extraction is usually non-selective and may be subject to more matrix effects than other extraction methods [22]. Good on column retention for the analytes was therefore needed to avoid matrix suppression. The use of labeled analytes as internal standards compensates for any matrix effects and further enhances the robustness of the method. The performance of this LC–MS/MS method was not compromised for the simultaneous analysis of four probes. Nevertheless, one must be aware that from the bioanalytical point of view, it is a disadvantage of including too many analytes/metabolites in a single assay. It not only increases significantly the cost of developing and validating such an assay but also inevitably imposes a significant assay failure risk. Failed run would not only need substantial investigation for root cause but also potentially generates duplicate sets of bioanalytical data for some probe substrates or metabolites. One must be judicial on validating assay suitable for cocktail analysis and labeled analyte as internal standard for each probe is highly recommended to mitigate risk of assay failure.

One must also be aware that due to lack of industrial consensus on the probe selection and the clinical preference of not dosing unnecessary probe substrates to healthy volunteers, a literature assay is rarely used as is for a new study. The current assay bears the same limitation. Any additional or subtraction from the current combination (for example addition a probe such as caffeine for CYP1A2) requires additional development and validation work.

The electrospray ionization gave the optimum sensitivity for tolbutamide, omeprazole, midazolam, and dextromethorphan in positive ion mode. Considering the four analytes of interest with different requirement on sensitivity to support the DDI study, the declustering potentials (DP), potential (EP), collision energy (CE), and collision cell exit potential (CXP) were carefully optimized in manual tuning mode to meet the need of supporting clinical sample analysis.

The Q1 mass spectrum of tolbutamide, omeprazole, midazolam, and dextromethorphan showed protonated molecular ions $[M+H]^+$ at $m/z$ 271, 346, 326 and 272, respectively. The product ion scan spectrum of $m/z$ 271 for tolbutamide, $m/z$ 346 for omeprazole, $m/z$ 326 for midazolam and $m/z$ 272 for dextromethorphan showed high abundance fragment ions at $m/z$ 172, 198, 291 and 171, respectively. The ion transitions of $m/z$ 271 → 172 for tolbutamide, $m/z$ 346 → 198 for omeprazole, $m/z$ 326 → 291 for midazolam, and $m/z$ 272 → 171 for dextromethorphan were chosen for multiple reaction monitoring (MRM).

### 3.2. Specificity, sensitivity and calibration linearity range

Human blank plasma samples from six different subjects were extracted and analyzed for TOL, OME, MID and DEX as a true blank (double blank), or spiked with ISs, or with the one of analytes as a single blank. There were no endogenous peaks that interfered with the quantitation of TOL, OME, MID, DEX or internal standards. There was no interference from internal standards contributing to the TOL, OME, MID and DEX $m/z$ channels or from TOL, OME, MID and DEX contributing to the IS $m/z$ channel. The interference between the new chemical entity (NCE) and tolbutamide, omeprazole, midazolam, and dextromethorphan at their respective retention times was also evaluated. No significant interference was detected at the retention time of any of the analytes with respect to the NCE. Good signal to noise ratios were achieved for all of the analytes. There was no significant lot-to-lot variation in matrix effect and no carry-over from ULOQ to blank sample observed. Calibration curves were well fit in the concentration range of 50/1/0.1/0.05 to 50,000/1000/100/50 ng/mL of TOL/OME/MID/DEX ng/mL using a linear regression with a weighting factor of the reciprocal of the concentration squared ($1/x^2$) for TOL, OME, MID and DEX. Representative chromatograms of blank human plasma spiked with TOL, OME, MID and DEX at LLOQ and IS are shown in Fig. 2.

### 3.3. Precision, accuracy and dilution integrity

Table 1 shows the validation data on accuracy and precision of each standard concentration. The coefficients of variation (CV, $N$ = 6) of the back-calculated calibration standards at 50/1/0.1/0.05 ng/mL of TOL/OME/MID/DEX was 2.0, 1.9, 1.0 and 6.0% for TOL, OME, MID and DEX, respectively, and at 50,000/1000/100/50 ng/mL of TOL/OME/MID/DEX was 2.5, 1.9, 1.2 and 1.6%, respectively. The precision and accuracy data for QCs are summarized in Table 2. For QCs at 50/1/0.1/0.05 ng/mL of TOL/OME/MID/DEX (LLOQ) and 39,000/780/78/39 ng/mL of TOL/OME/MID/DEX (high QC), inter-assay CV values were 10.2 and 2.1%, respectively, for TOL, 7.3 and 2.3%, respectively, for OME, 1.9 and 2.1%, respectively, for MID, and 14.3 and 2.2%, respectively, for DEX. The %Nominal at LLOQ were 100.0% for TOL, 95.9% for OME, 103.0% for MID, and 98.0% for DEX, respectively, indicating reliable quantitation at the LLOQ level. The "tight" CV and %Nominal values indicated reproducible LC–MS/MS conditions and that the assay is consistent and reliable. For partial volume analysis, QC samples (100,000/2000/200/100 ng/mL of TOL/OME/MID/DEX) were diluted 20-fold with blank plasma prior to extraction. The dilution integrity (20-fold dilution) showed the CV was 2.6, 2.7, 2.8 and 2.8% for TOL, OME, MID and DEX, respectively, with a %Nominal of 106.9, 99.1, 95.8 and 92.2% for TOL, OME, MID and DEX, respectively. These results support sample dilution up to 20-fold for analysis.

### 3.4. Recovery and matrix effect

The overall extraction recovery was determined by comparing the peak areas of extracted plasma standards at low, mid and high QC levels to the peak areas of neat solutions spiked at corresponding concentrations. Extraction recovery from human plasma ranged from 97.4 to 99.5% for TOL, 89.7 to 102.2% for OME, 92.8 to 103.1% for MID, and 89.0 to 105.0% for DEX, respectively. Here, the combined contributions from recovery loss and matrix suppression were measured. These results indicated excellent recovery and minimal matrix effects. In addition, the post-column infusion experiment was performed and confirmed the minimal matrix effects in this 4-in-1 method.

### 3.5. Stability of the analytes

The stability tests were designed to cover the anticipated conditions that the clinical samples may experience. Stability of sample processing (freeze/thaw and bench-top) and reinjection of processed samples were tested and established. Stability data are summarized in Table 3. Briefly, three freeze/thaw cycles and ambi-

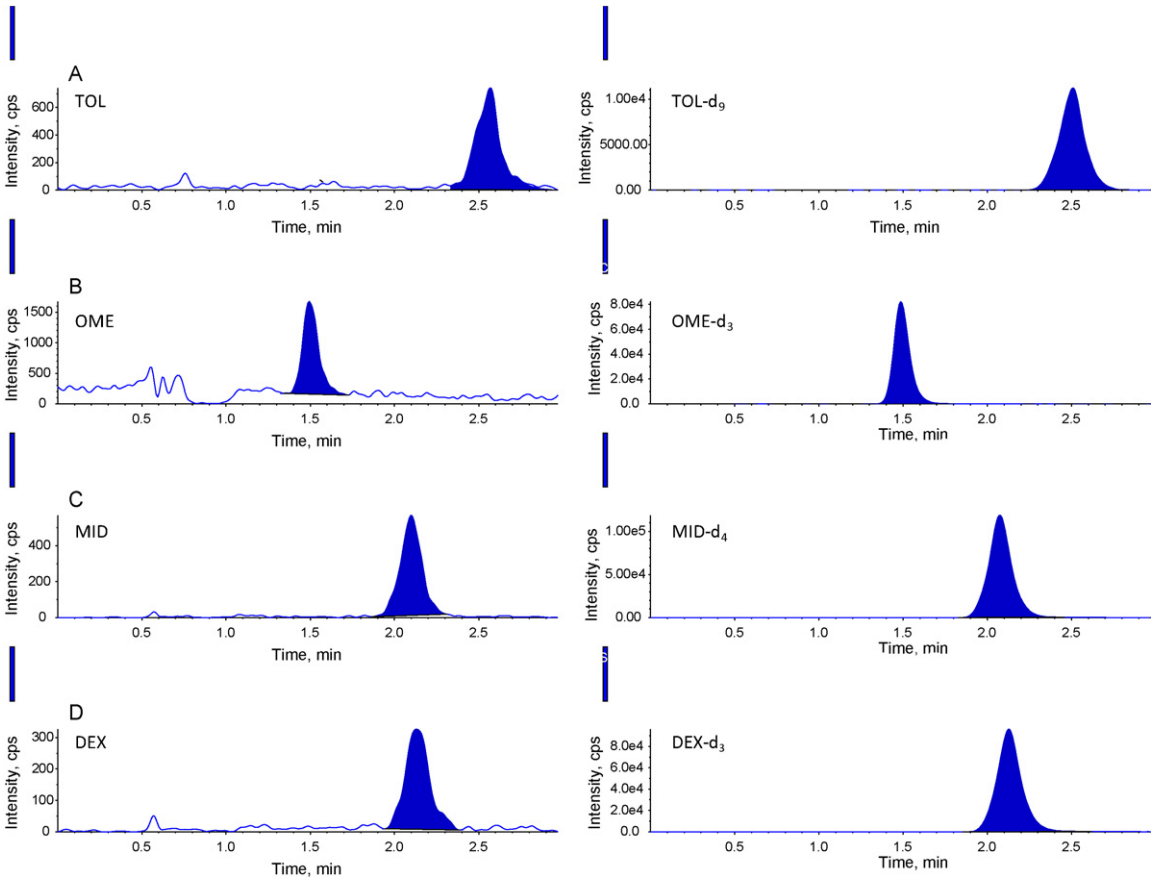


**Fig. 2.** LC–MS/MS chromatograms of tolbutamide (A), omeprazole (B), midazolam (C), and dextromethorphan (D) at the low limit of quantitation (50/1/0.1/0.05 ng/mL for TOL/OME/MID/DEX in human plasma).

**Table 1**
Precision and accuracy of calibration standards ($N = 6$).

| $N = 6$ | Tolbutamide, ng/mL | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 50 | 100 | 500 | 2500 | 5000 | 20,000 | 40,000 | 50,000 |
| Mean | 50.2 | 98.8 | 504 | 2577 | 5098 | 20,338 | 38,767 | 48,125 |
| SD | 0.999 | 4.12 | 14.9 | 44.4 | 112 | 457 | 827 | 1217 |
| %CV | 2.0 | 4.2 | 3.0 | 1.7 | 2.2 | 2.2 | 2.1 | 2.5 |
| %Nominal | 100.4 | 98.8 | 100.8 | 103.1 | 102.0 | 101.7 | 96.9 | 96.2 |
| $N = 6$ | Omeprazole, ng/mL | | | | | | | |
| | 1 | 2 | 10 | 50 | 100 | 400 | 800 | 1000 |
| Mean | 1.01 | 1.95 | 10.3 | 52.2 | 1037 | 4022 | 771 | 943 |
| SD | 0.019 | 0.077 | 0.156 | 0.532 | 1.41 | 7.29 | 21.5 | 17.8 |
| %CV | 1.9 | 4.0 | 1.5 | 1.0 | 1.4 | 1.8 | 2.8 | 1.9 |
| %Nominal | 101.0 | 97.3 | 102.8 | 104.5 | 103.3 | 100.6 | 96.3 | 94.3 |
| $N = 6$ | Midazolam, ng/mL | | | | | | | |
| | 0.1 | 0.2 | 1 | 5 | 10 | 40 | 80 | 100 |
| Mean | 0.100 | 0.198 | 0.989 | 5.07 | 10.1 | 40.3 | 79.3 | 99.0 |
| SD | 0.001 | 0.004 | 0.015 | 0.083 | 0.123 | 0.547 | 1.38 | 1.22 |
| %CV | 1.0 | 2.0 | 1.5 | 1.6 | 1.2 | 1.4 | 1.7 | 1.2 |
| %Nominal | 100.0 | 99.0 | 98.9 | 101.4 | 101.4 | 100.6 | 99.0 | 99.0 |
| $N = 6$ | Dextromethorphan, ng/mL | | | | | | | |
| | 0.05 | 0.1 | 0.5 | 2.5 | 5 | 20 | 40 | 50 |
| Mean | 0.050 | 0.102 | 0.499 | 2.48 | 5.01 | 20.1 | 39.9 | 49.8 |
| SD | 0.003 | 0.011 | 0.008 | 0.048 | 0.028 | 0.598 | 0.907 | 0.791 |
| %CV | 6.0 | 10.8 | 1.6 | 1.9 | 0.6 | 3.0 | 2.3 | 1.6 |
| %Nominal | 100.0 | 102.0 | 99.8 | 99.4 | 100.2 | 100.5 | 99.7 | 99.6 |

**Table 2**
Precision and accuracy of quality control samples.

| Day ID | N = 6 | Tolbutamide, ng/mL | | | Omeprazole, ng/mL | | | Midazolam, ng/mL | | | Dextromethorphan, ng/mL | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 150 | 4000 | 39,000 | 3 | 80 | 780 | 0.3 | 8 | 78 | 0.15 | 4 | 39 |
| Intraday 1 | Mean | 163 | 3997 | 35,243 | 3.22 | 81.6 | 712 | 0.330 | 7.97 | 73.2 | 0.162 | 3.97 | 36.7 |
| | SD | 6.73 | 56.1 | 542 | 0.090 | 1.76 | 16.5 | 0.017 | 0.161 | 1.86 | 0.009 | 0.089 | 0.763 |
| | %CV | 4.1 | 1.4 | 1.5 | 2.8 | 2.2 | 2.3 | 5.2 | 2.0 | 2.5 | 5.6 | 2.2 | 2.1 |
| | %Nominal | 108.8 | 99.9 | 90.4 | 107.3 | 101.9 | 91.3 | 110.0 | 99.7 | 93.9 | 108.0 | 99.2 | 94.1 |
| Intraday 2 | Mean | 159 | 4023 | 36,297 | 3.14 | 82.6 | 728 | 0.307 | 7.92 | 73.5 | 0.161 | 3.93 | 36.6 |
| | SD | 8.02 | 64.0 | 627 | 0.117 | 1.25 | 18.8 | 0.012 | 0.094 | 1.19 | 0.005 | 0.077 | 0.607 |
| | %CV | 5.1 | 1.6 | 1.7 | 3.7 | 1.5 | 2.6 | 3.9 | 1.2 | 1.6 | 3.1 | 2.0 | 1.7 |
| | %Nominal | 105.9 | 100.6 | 93.1 | 104.6 | 103.2 | 93.3 | 102.3 | 99.0 | 94.2 | 107.3 | 98.3 | 93.9 |
| Intraday 3 | Mean | 151 | 4094 | 35,407 | 3.14 | 83.6 | 716 | 0.299 | 8.25 | 74.2 | 0.155 | 4.16 | 37.4 |
| | SD | 8.96 | 74.6 | 600 | 0.084 | 1.87 | 11.4 | 0.010 | 0.133 | 1.52 | 0.006 | 0.158 | 0.908 |
| | %CV | 5.9 | 1.8 | 1.7 | 2.7 | 2.2 | 1.6 | 3.3 | 1.6 | 2.0 | 3.9 | 3.8 | 2.4 |
| | %Nominal | 100.7 | 102.3 | 90.8 | 104.6 | 104.5 | 91.8 | 99.7 | 103.2 | 95.1 | 103.3 | 103.9 | 95.8 |
| Interday results | Mean | 158 | 4038 | 35,649 | 3.17 | 82.6 | 719 | 0.312 | 8.05 | 73.6 | 0.159 | 4.02 | 36.9 |
| | SD | 9.09 | 74.4 | 732 | 0.100 | 1.78 | 16.4 | 0.019 | 0.194 | 1.51 | 0.007 | 0.146 | 0.801 |
| | %CV | 5.8 | 1.8 | 2.1 | 3.2 | 2.2 | 2.3 | 6.1 | 2.4 | 2.1 | 4.4 | 3.6 | 2.2 |
| | %Nominal | 105.1 | 101.0 | 91.4 | 105.5 | 103.2 | 92.1 | 104.0 | 100.6 | 94.4 | 106.0 | 100.5 | 94.6 |

ent temperature storage of the QC samples up to 6 h prior to sample preparation appeared to have no effect on the quantitation of TOL, OME, MID and DEX. QCs stored in a freezer at −20 °C remained stable for at least 90 days. Extracted analytes were allowed to stand at ambient temperature in mobile phase for 46 h prior to LC–MS/MS analysis, with no observed effect on quantitation. Stability of stock solutions was also investigated. When stock solutions of TOL, OME, MID and DEX in a in methanol/water (50:50, v/v) except for omeprazole in methanol/water/50% NaOH with ~0.36% of $Na_2CO_3$ (20/50/0.2, v/v/v) were stored at a nominal temperature of 4 °C for 2 months or at room temperature for 6 h, the analytes were stable. The stabilities of TOL, OME, MID and DEX in whole blood were evaluated at two temperatures (ice bath, wet ice, 0–4 °C and room temperature), three time periods (0, 30, and 120 min) and two concentration levels (150/3/0.3/0.15 ng/mL (low QC) and 39,000/780/78/39 ng/mL (high QC) for TOL/OME/MID/DEX, respectively). The mean area ratio for TOL, OME, MID and DEX at all other time-points were agreeable with the mean area ratio at time 0 h (Difference was <15%). Therefore, it can be concluded that TOL, OME, MID and DEX are stable in whole blood on ice bath (wet ice, 0–4 °C) and at room temperature for at least 2 h.

In summary, the full validation of the method was carried out in compliance with the lab SOP—General Guidelines for the Validation of Bioanalytical Methods. All data reported in this validation met the method validation acceptance criteria defined in SOP and fulfilled the requirements and recommendations in the FDA guidance for bioanalytical method validations. All the validation runs, conducted on 14Dec08, 15Dec08, 16Dec08, 17Dec08, 31Jan09, 02Feb09, 12Feb09, and 12Mar09 were successfully completed.

### 3.6. Method robustness and incurred sample reproducibility (ISR)

There are some published LC–MS/MS methods combined with in vivo or in vitro cocktail approaches reported, but none of them included the same four-specific probe substrates. Most of the methods were not fully validated according to the FDA bioanalytical method validation guidance. The method presented in this manuscript was optimized to meet the sample analysis requirements and fully validated according to the guidance. The validated LC–MS/MS method has successfully been used to support sample analysis of a clinical drug–drug interaction study with excellent QC performance (CV < 7.5%; accuracy: 92.6–103.1%) and 100% pass rate in a total of 24 analytical runs. The representative chromatograms of an extracted human plasma sample (Subject 1001 B, Day 5, 2 h) are presented in Fig. 3.

Fifty clinical study samples for tolbutamide, omeprazole, midazolam, and dextromethorphan were selected per the Incurred Sample Reproducibility Study Plan. The results, presented in Fig. 4, met the acceptance criteria. The criteria specify that the %Difference, as calculated below, of 2/3 of all the analyzed ISR samples at least should be within ±20%. The overall ISR results for all compounds showed that over 95% of the samples had a %Diff of less than 10% and demonstrated that method was reproducible and robust.

**Table 3**
Stability of tolbutamide, omeprazole, midazolam and dextremethorphan.

| Stability | N = 6 | Tolbutamide, ng/mL | | | Omeprazole, ng/mL | | | Midazolam, ng/mL | | | Dextromethorphan, ng/mL | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 150 | 4000 | 39,000 | 3 | 80 | 780 | 0.3 | 8 | 78 | 0.15 | 4 | 39 |
| Bench-top | Mean | 155 | 4093 | 34,599 | 3.09 | 84.1 | 701 | 0.312 | 8.22 | 71.9 | 0.152 | 4.08 | 36.2 |
| | SD | 4.28 | 85.0 | 202 | 0.087 | 0.684 | 7.53 | 0.004 | 0.130 | 0.836 | 0.008 | 0.063 | 0.558 |
| | %CV | 2.8 | 2.1 | 0.6 | 2.8 | 0.8 | 1.1 | 1.3 | 1.6 | 1.2 | 5.3 | 1.5 | 1.5 |
| | %Nominal | 103.3 | 102.3 | 88.7 | 103.0 | 105.1 | 89.9 | 104.0 | 102.7 | 92.1 | 101.3 | 102.0 | 92.9 |
| Freeze/thaw (three cycles) | Mean | 154 | 4124 | 35,709 | 3.25 | 83.50 | 720 | 0.298 | 8.26 | 74.0 | 0.156 | 4.15 | 36.4 |
| | SD | 7.36 | 78.1 | 603 | 0.138 | 1.053 | 7.91 | 0.008 | 0.154 | 0.366 | 0.014 | 0.070 | 0.402 |
| | %CV | 4.8 | 1.9 | 1.7 | 4.3 | 1.3 | 1.1 | 2.7 | 1.9 | 0.5 | 9.0 | 1.7 | 1.1 |
| | %Nominal | 102.4 | 103.1 | 91.6 | 108.2 | 104.4 | 92.3 | 99.3 | 103.3 | 94.8 | 104.0 | 103.7 | 93.4 |
| Processed sample stability (46 h) | Mean | 154 | 3976 | 34,994 | 3.14 | 81.8 | 709 | 0.297 | 8.04 | 73.6 | 0.154 | 4.00 | 37.1 |
| | SD | 6.14 | 71.2 | 509 | 0.070 | 1.16 | 13.0 | 0.006 | 0.089 | 0.941 | 0.006 | 0.049 | 0.740 |
| | %CV | 4.0 | 1.8 | 1.5 | 2.2 | 1.4 | 1.8 | 2.0 | 1.1 | 1.3 | 3.9 | 1.2 | 2.0 |
| | %Nominal | 103.0 | 99.4 | 89.7 | 104.7 | 102.2 | 90.9 | 99.0 | 100.5 | 94.3 | 102.7 | 100.0 | 95.0 |

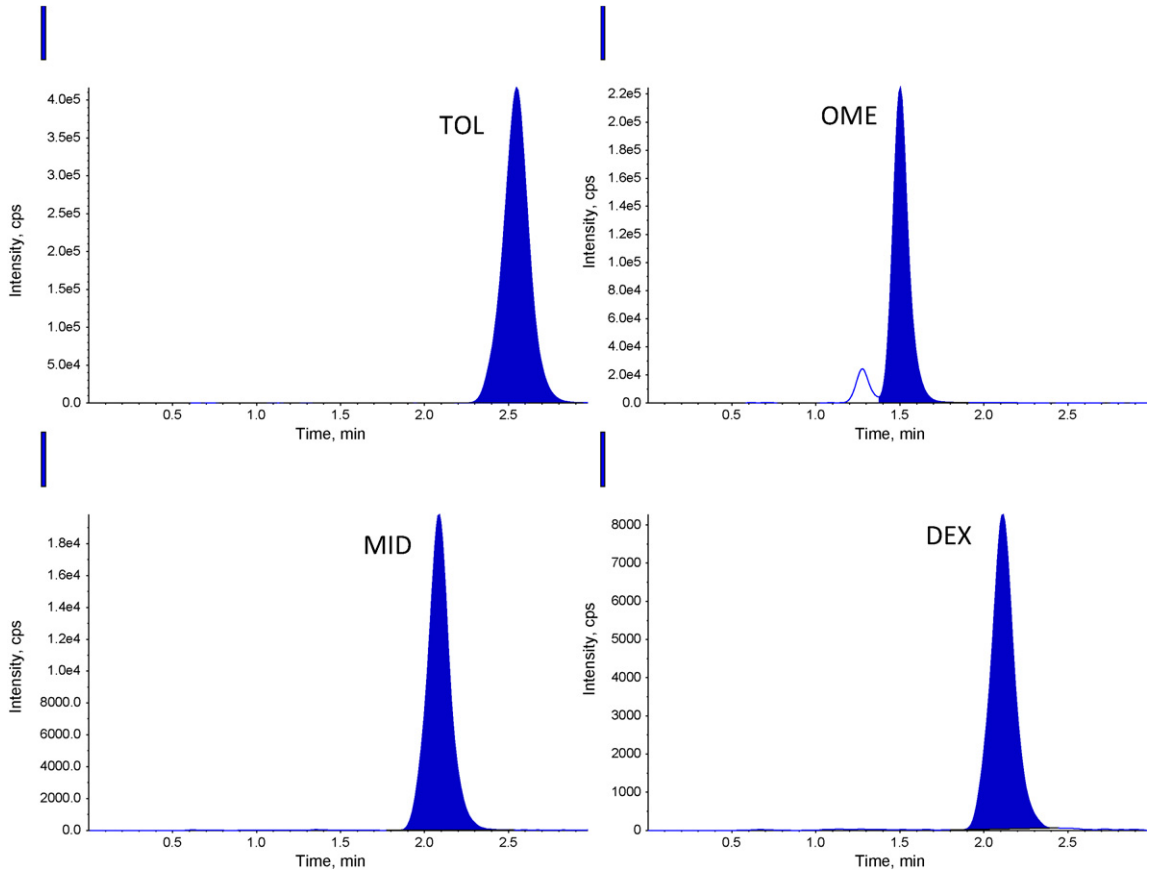


**Fig. 3.** The representative chromatograms of an extracted clinical human plasma sample (Subject 1001 B, Day 5, 2 h) with determined concentrations of TOL/OME/MID/DEX at 30,597/207/4.30/1.97 ng/mL, respectively.

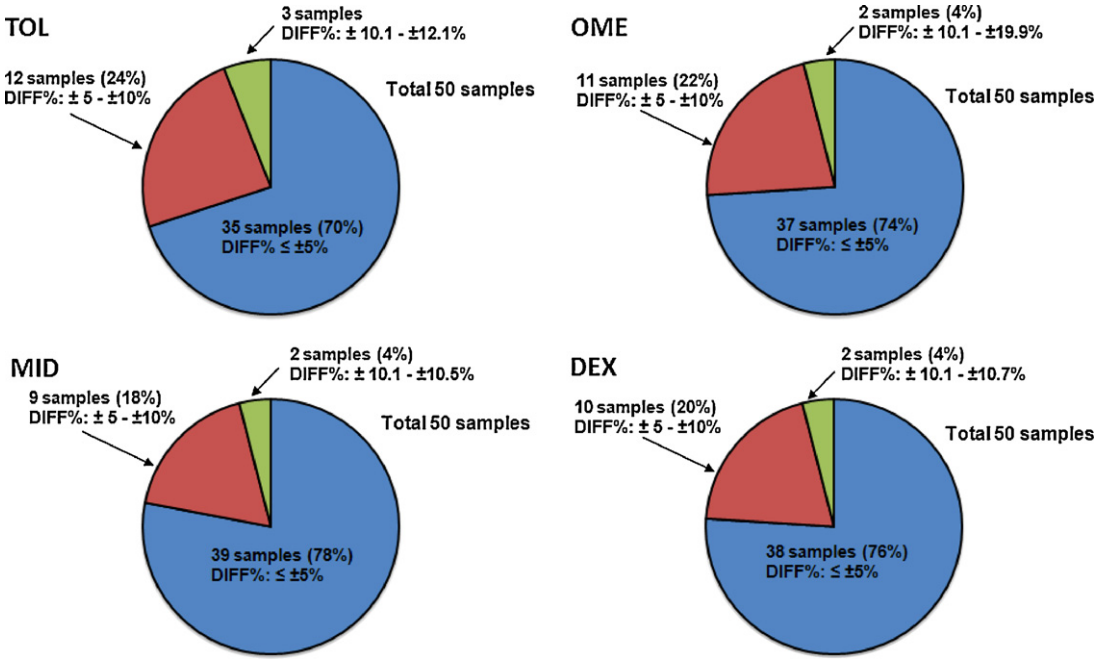


**Fig. 4.** Results of incurred sample reproducibility (ISR) test of 50 selected clinical study samples.

Calculation for %Difference:

$$\%\text{Difference} = \frac{\text{repeat value} - \text{original value}}{\text{mean of original value and repeat value}} \times 100$$

## 4. Conclusions

An LC–MS/MS method for simultaneous determination of four marker substrates of cytochrome P450, namely tolbutamide (CYP2C9), omeprazole (CYP2C19), midazolam (CYP3A4), and dextromethorphan (CYP2D6) in plasma, over the concentration range of 50/1/0.1/0.05 to 50,000/1000/100/50 ng/mL of TOL/OME/MID/DEX using 50 μL sample size, has been successfully developed and validated. The validation study successfully evaluated intraday, interday, selectivity, sensitivity, linearity, recovery, dilution integrity, processed stability, bench-top, freeze/thaw stability, stock solution stability, long-term sample stability, and whole blood stability in the above stated concentration range for tolbutamide, omeprazole, midazolam, and dextromethorphan. The 100% pass rate from the 24 sample analysis runs supporting the clinical study and the ISR evaluation results demonstrated that method was reproducible and robust. The method is simple, rapid and rugged, and is suitable for routine quantitation of tolbutamide, omeprazole, midazolam, and dextromethorphan in human plasma.

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