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Validation of a Sensitive UHPLC-MS/MS Method for Cytochrome P450 Probe Substrates Caffeine, Tolbutamide, Dextromethorphan, and Alprazolam in Human Serum Reveals Drug Contamination of Serum Used for Research

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Abstract

To evaluate the potential for interactions between botanical dietary supplements and drug metabolism, Phase I clinical pharmacokinetics studies are conducted using an oral cocktail of probe substrates of cytochrome P450 (CYP) enzymes. A sensitive, specific, and fast ultra-high performance liquid chromatography/tandem mass spectrometry (UHPLC-MS/MS) method was developed and validated for determination of caffeine (probe of CYP1A2), tolbutamide (probe of CYP2C9), dextromethorphan (probe of CYP2D6), and alprazolam (probe of CYP3A4/5) in human serum. Stable isotope-labelled analogs were used as internal standards, and sample preparation involved only rapid protein precipitation and centrifugation. The method of standard addition was used for the measurement of caffeine, because commercially available pooled human serum contains caffeine. Out of 18 lots of pooled human serum tested, caffeine was detected in all lots, alprazolam was detected in 13 lots, 8 lots contained dextromethorphan, and no tolbutamide was detected. Only serum prepared from the blood of select individuals was determined to be drug-free. The analytical method was validated with respect to linearity, accuracy and precision, recovery, stability, and matrix effects. The calibration curves were linear over the range of 25-12,000 ng/mL for caffeine, 75-36,000 ng/mL for tolbutamide, 0.05-30 ng/mL for dextromethorphan, and 0.1-60 ng/mL for alprazolam. The intra-assay and inter-assay coefficients of variation (%CV) and %Bias were <13% (<17% at the lower limit of quantitation). The recovery of each probe substrate ranged from 84.2% to 98.5%. All analytes were stable during sample storage and handling. Matrix effects were minimized by using stable isotope-labeled internal standards. The method was successfully applied to clinical studies investigating the

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pharmacokinetic alterations of probe substrates caused by chronic consumption of botanical dietary supplements.

Graphical abstract

Keywords

liquid chromatography; mass spectrometry; cytochrome P450; probe substrates

1. Introduction¹

Due to the combination speed, sensitivity and selectivity, liquid chromatography-mass spectrometry (LC-MS) and tandem mass spectrometry (MS/MS), have become essential for all stages of drug discovery and development. An important step in establishing the safety of new drugs is to evaluate the potential for drug-drug pharmacokinetic interactions. As the market grows for botanical dietary supplements, the potential for drug-botanical interactions is becoming increasingly important [1,2]. Mechanisms of drug-drug and drug-botanical interactions can include inhibition and/or induction of cytochrome P450 (CYP) enzymes.

A family of heme-containing proteins expressed in many organs, CYP enzymes are highly concentrated in human liver and are responsible for the metabolism and clearance of most clinically used drugs [3]. Induction or inhibition of CYP by drugs or botanical dietary supplements can alter the pharmacokinetics of other therapeutic agents. A well-documented example of a drug-botanical interaction involves the botanical dietary supplement St John's Wort, which induces the most abundant of the CYP, CYP3A4. As a result of induction, therapeutic agents metabolized by CYP3A4, such the HIV-1 protease inhibitor indinavir, will be metabolized and eliminated more rapidly [4]. Alternatively, inhibition of a specific CYP will extend the half-life and exposure to drugs metabolized by this enzyme [1].

The definitive approach to evaluate drug-drug and drug-botanical pharmacokinetic interactions involves clinical investigation of the extent to which the test agent induces or inhibits drug metabolizing enzymes and transporters. In most clinical studies, the alterations in enzymatic activity of CYP are determined by the differences in serum levels of probe

¹Abbreviations: S/N: signal over noise; CYP: cytochrome P450; ULOQ: upper limit of quantitation.

substrates over time due to consumption of the test drug or botanical dietary supplement [5]. Typically, a mixture of probe substrates is administered orally in a single dose drug cocktail, serial blood samples are drawn, measured using LC-MS/MS, and pharmacokinetic parameters are calculated for each probe [5]. The same study subjects then consume the test drug or botanical dietary supplement for at least 14 days to allow for both induction as well as inhibition of CYP, and then the probe substrate cocktail experiment is repeated. Changes in the pharmacokinetic parameters of each probe substrate would indicate a drug-drug or drug-botanical interaction.

For clinical studies of drug-drug and drug-botanical interactions, LC-MS/MS has become standard for measuring serum or plasma levels of the probe substrates [6-9]. To support a series of clinical trials of drug-botanical dietary supplement interactions, we developed and validated an ultra-high pressure LC-MS/MS (UHPLC-MS/MS) assay to measure serum concentrations of caffeine, tolbutamide, dextromethorphan, and alprazolam (Figure 1) in a cocktail of probe substrates for CYP1A2, CYP2C9, CYP2D6, and CYP3A4/5, respectively. None of these probe substrates are reported to be CYP inhibitors or inducers, and all are recommended by the U.S. Food and Drug Administration (FDA) for clinical drug-drug interaction research [10]. To complement the speed, sensitivity and selectivity of LC-MS/MS, UHPLC was used to enhance the throughput of the assays, and sample preparation was simple and fast, involving addition of organic solvent to precipitate serum proteins followed by centrifugation. Not reported in previous studies of drug interactions using probe substrates, obtaining drug-free serum for the preparation of standard curves posed a significant challenge.

2. Material and methods

2.1 Chemicals and Reagents

Caffeine, [trimethyl- $^{13}\text{C}_3$]-caffeine, tolbutamide, dextromethorphan, [methyl- d_3]-dextromethorphan, alprazolam, and [phenyl- d_5]-alprazolam were purchased from Sigma-Aldrich (St. Louis, MO), and [butyl- d_9]-4-hydroxy-tolbutamide was purchased from Toronto Research Chemicals (Toronto, Canada) (Figure 1). LC-MS grade acetonitrile and methanol were purchased from VWR (Radnor, PA), and LC-MS grade formic acid was purchased from Thermo Scientific (Rockford, IL). Water was prepared using an Elga Purelab Ultra (Siemens Water Technologies, Woodridge, IL) water purification system.

Pooled human serum and serum from individual donors were purchased from Valley Biomedical Products & Services (Winchester, VA), Innovative Research (Novi, MI), Sigma-Aldrich (St. Louis, MO), and BioIVT (Westbury, NY). Serially drawn serum samples from women who had been administered an oral cocktail of 100 mg caffeine, 250 mg tolbutamide, 30 mg dextromethorphan, and 2 mg alprazolam were obtained from the UIC/NIH Center for Botanical Dietary Supplements Research (registered with [ClinicalTrials.gov](https://clinicaltrials.gov) as , UIC Institutional Review Board #2015-0651). These serum samples contained no patient identifying information. All serum was stored at $-80\text{ }^{\circ}\text{C}$ until use.

2.2 Sample preparation

For the measurement of caffeine and tolbutamide, 50 μL aliquots of serum human serum were thawed and vortex mixed with 50 μL aliquots of water containing 0.1% formic acid. Protein precipitation was carried out by adding to each sample 300 μL of acetonitrile/methanol (9:1, v/v) containing 1,000 ng/mL of [$^{13}\text{C}_3$]-caffeine and 2,400 ng/mL of [d_9]-4-hydroxy-tolbutamide as internal standards. The mixture was vortexed for 0.5 min and centrifuged for 15 min at $13000 \times g$ at 4 $^\circ\text{C}$. Each supernatant (80 μL) was transferred to a new Eppendorf tube and diluted with 20 μL of 30% aqueous methanol containing 0.1% formic acid.

For the measurement of dextromethorphan and alprazolam, aliquots (100 μL) of human serum were spiked with 100 μL aliquots of water containing 0.1% formic acid. Protein precipitation was carried out by adding to each sample 600 μL of acetonitrile/methanol (9:1, v/v) containing 28 ng/mL of [d_3]-dextromethorphan and 15 ng/mL of [d_5]-alprazolam as internal standards. Each mixture was vortexed for 0.5 min and centrifuged for 15 min at $13000 \times g$ at 4 $^\circ\text{C}$. The supernatant was transferred to a new Eppendorf tube and evaporated to dryness. The residue was reconstituted in 50 μL of 30% aqueous methanol containing 0.1% formic acid, vortexed and centrifuged as described above, and the supernatant was transferred to an autosampler vial for analysis using UHPLC-MS/MS.

2.3 Calibration curves and quality controls

At a final concentration of 10 mg/mL, caffeine stock solution was prepared in water, and tolbutamide stock solution was prepared in methanol. Both solutions were stored in amber glass vials. Working standards were prepared by serial dilution from stock solutions using 50% aqueous methanol. Quality control (QC) stock solutions were prepared from a separate weighing of the standards. Calibration standards and QC samples were prepared by mixing 5 μL of each working standard or QC solution with 45 μL blank serum. The concentrations of caffeine used for the standard curve were 25, 50, 100, 200, 500, 1000, 4000, 8000, and 12000 ng/mL, and those for tolbutamide were 75, 150, 300, 600, 1500, 3000, 12000, 24000, and 36000 ng/mL. Protein precipitation was carried out as described in section 2.2 Sample preparation.

Dextromethorphan and alprazolam stock solutions were prepared in methanol at a final concentration of 1 mg/mL. Storage and working solution preparation were as described above. Calibration standards and QC samples were prepared by mixing 5 μL of each working standard or QC solution with 95 μL blank serum. The concentrations of dextromethorphan used for the standard curve were 0.05, 0.1, 0.25, 0.5, 1, 5, 15, and 30 ng/mL, and those for alprazolam were 0.1, 0.2, 0.5, 1, 2, 10, 30, and 60 ng/mL. Protein precipitation was carried out as described in section 2.2 Sample preparation.

2.4 UHPLC-MS/MS

The probe substrates were measured using a Shimadzu (Kyoto, Japan) Nexera UHPLC system interfaced to a Shimadzu LCMS-8060 triple quadrupole mass spectrometer (MS/MS). Separations were carried out using a Waters (Milford, MA) ACQUITY UHPLC BEH (2.1×50 mm, 1.7 μm) C_{18} column with a gradient from water (containing 0.1% formic

acid) to acetonitrile as follows: 5-15% acetonitrile 0-0.7 min, 15-55% acetonitrile 0.7-1.5 min, 55-75% acetonitrile from 1.5-2.5 min, and 1 min at 95% acetonitrile, followed by re-equilibration at 5% acetonitrile for 1 min. The total run time including equilibration was 4.5 min. The flow rate was 0.5 mL/min, the column oven temperature was 40 °C, and the autosampler temperature was 4 °C.

Positive ion electrospray mass spectrometry with collision-induced dissociation and selected reaction monitoring (SRM) was used for quantitative analysis. Mass spectrometer parameters were optimized for each probe substrate using flow injection. Data were acquired by monitoring 2 SRM transitions (quantifier and qualifier) for each analyte as follows: caffeine: m/z 195 to 138 and m/z 195 to 110; [$^{13}\text{C}_3$]-caffeine: m/z 198 to 140 and m/z 198 to 112; tolbutamide: m/z 271 to 172 and m/z 271 to 91; [d_9]-4-hydroxy-tolbutamide: m/z 296 to 188 and m/z 296 to 107; dextromethorphan: m/z 272 to 215 and m/z 272 to 171; [d_3]-dextromethorphan: m/z 275 to 215 and m/z 275 to 171; alprazolam: m/z 309 to 281 and m/z 309 to 205; [d_5]-alprazolam: m/z 314 to 286 and m/z 314 to 210; and ring-opened alprazolam: m/z 327 to 310 and m/z 327 to 298. The SRM dwell time was 25 ms/ion. The desolvation line temperature was 250°C, the nebulizing gas flow was 3 L/min, and the drying gas flow was 10 L/min. Data acquisition and quantitative analysis were carried out using Shimadzu LabSolutions software.

2.5 Method validation

Method validation was carried out according to the FDA guidance for industry on bioanalytical method validation [11] with respect to linearity, specificity, accuracy and precision, extraction recovery, stability, and matrix effect.

2.5.1 Selectivity and specificity—Blank human serum from 6 different commercial lots or individuals was tested for interference to assess the specificity of the method at the lower limit of quantitation (LLOQ). The LLOQ was defined as a signal to noise (S/N) ratio of 10.

2.5.2 Precision and accuracy—The accuracy and precision of the assay were assessed by analyzing 5 replicates at LLOQ, as well as low, medium, and high QC samples. Calibration curves were evaluated for 3 different analytical runs on 3 separate days. The accuracy was calculated as the ratio of the mean value of the QC samples to the true value. The precision was expressed as the coefficient of variation (%CV). Chromatographic carryover was assessed by comparing the peak areas of analytes at the LLOQ to the corresponding peak areas for blank injections made immediately after injecting analytes at the upper limit of quantitation (ULOQ).

2.5.3 Recovery—The recoveries of each analyte at low, medium, and high QC concentrations were determined 3 times and were calculated by comparing the analyte/internal standard SRM MS/MS responses of the extracted samples with the responses of blank matrix spiked with analyte after extraction. For recovery measurements, internal standards were spiked into the samples immediately before analysis using UHPLC-MS/MS.

2.5.4 Matrix effect—The potential for matrix effects was evaluated by spiking analytes and internal standards into extracted matrix blanks obtained from 6 different lots of human serum and then comparing the peak areas of the post-extraction spiked samples with the peak areas of low and high QC concentrations. For dextromethorphan, the medium QC concentration was used instead of the high concentration.

2.5.5 Stability—Analyte stabilities at low and high concentrations were determined in stock solutions as well as in extracted QC samples. Short-term stability was evaluated for QC samples maintained on the bench-top at room temperature as well as in an autosampler at 4 °C for 24 h. Freeze-thaw stabilities were assessed by freezing samples at –20 °C followed by thawing at room temperature for 3 cycles. Long-term stabilities were investigated for QC samples stored at –80 °C and for stock solutions frozen at –20 °C for up to 4 months. The stabilities were determined by comparing the concentrations of treated standards or QC samples with their concentrations at zero time (for bench-top and autosampler samples) or with freshly prepared samples (for freeze-thaw, long-term QC and stock solutions). During stability testing, 3 replicates of QC samples were prepared for each concentration under each test condition, and 5 replicate UHPLC-MS/MS analyses of standards were carried out for each stock solution.

3. Results and Discussion

3.1 Method development

Stable isotopically-labeled internal standards were used to enhance the accuracy and precision of the UHPLC-MS/MS assays of all probe substrates. Due to lack of commercially available stable isotopically-labelled tolbutamide, its deuterated metabolite, [d₉]-4-hydroxy-tolbutamide, was used as an internal standard instead. The serum concentrations of caffeine and tolbutamide spanned four orders of magnitude, while those of dextromethorphan and alprazolam were only one order of magnitude. The calibration range and stock solution concentrations of each probe substrate were adjusted accordingly.

For a typical clinical study of drug-botanical interactions, serum or plasma is obtained at multiple time points (in our studies at 36 time points) from a minimum of 16 subjects. Due to the large number of samples, protein precipitation was used to partially purify serum prior to UHPLC-MS/MS analysis due to its speed, simplicity and low cost [12]. Despite these advantages, liquid/liquid extraction [13] or solid phase extraction [6,7] is still used most often for drug-drug and drug-botanical interaction studies. In addition, most of the drug interaction studies that report using organic solvent to precipitate serum/plasma proteins have utilized methanol [8,9], even though acetonitrile is more effective for protein removal [14]. In our study, acetonitrile was used for protein precipitation, and a low proportion of methanol (acetonitrile/methanol, 9:1; v/v) was included to solubilize and recover the polar probe substrate caffeine. In addition, serum was acidified before protein precipitation to enhance the recovery of dextromethorphan from 50% to 85% (Table 1).

Optimized MS/MS parameters for each analyte are summarized in Table 2. Positive ion electrospray was selected due to better signal response and lower LLOQ values than were obtained in negative ion mode. The most abundant product ion for each protonated probe

substrate was used as the quantifier SRM transition, and the next most abundant product ion served as the qualifier. Because tolbutamide displayed a strong UHPLC-MS/MS signal response and required a higher upper limit of quantitation than the other probe substrates, a suboptimal collision-induced dissociation energy was used for the tolbutamide SRM quantifier to avoid signal saturation.

Different reversed phase solvents, mobile phase modifiers and gradients were evaluated for the separation of the 4 probe substrates. A mobile phase gradient of increasing acetonitrile in 0.1% aqueous formic acid provided separation of the probe substrates in less than 3 min (Figure 2). Including column re-equilibration, the total UHPLC-MS/MS run time was 4.5 min per sample. For comparison, most previous methods require from 11 to 18 min per run [6-8].

Among the probe substrates included in this method, only alprazolam had been reported to be sensitive to heat, light and acidic solvents [15-17]. Possible degradation products of alprazolam including triazolaminoquinoline, 8-hydroalprazolam, triazolaminoquinoline, and a ring-opened form were monitored using UHPLC-MS/MS [18,19] in serum and in extracts after exposure to laboratory light and acidified solvent for up to 16 hours. In addition to alprazolam, only ring-opened alprazolam was detected in all samples. As reported previously [15], alprazolam is in equilibrium with its ring-opened form under aqueous conditions. Cho *et al.* [20] studied the kinetics and equilibrium of this reversible alprazolam ring-opening reaction, and under similar conditions to those used here, equilibrium was reached in <20 min at a ratio of alprazolam/ring-opened alprazolam of ~80:20. Therefore, intact alprazolam was measured but not the ring-opened form, and this approach was found to produce accurate results with sufficient sensitivity.

3.2 Method validation

In total, 20 different lots of pooled and single donor human serum were purchased from various vendors and tested using UHPLC-MS/MS for background levels of alprazolam, caffeine, dextromethorphan, and tolbutamide. Surprisingly, alprazolam was detected in 13 lots of human serum, 18 lots contained caffeine, 8 lots contained dextromethorphan, but no tolbutamide was detected (Figure 3). These observations were not caused by carryover, because blank injections between analyses showed no analyte peaks. Furthermore, multiple injections of the same lot of human serum showed constant, instead of decreasing, peak areas for alprazolam, caffeine and dextromethorphan. Concentrations of these compounds in commercially available human serum for research were as high as 250 ng/mL for caffeine, 0.04 ng/mL for dextromethorphan, and 0.1 ng/mL for alprazolam. Because only 2 lots of serum, which were obtained from individual donors (BioIVT; Westbury, NY), contained no detectable caffeine (Figure 3), the method of standard addition [21] was used to determine caffeine concentrations in pooled serum. Note that the method validation results reported below included a background signal of caffeine.

Anticipating caffeine in the blood supply, Wohlfarth *et al.* [7] drew blood from individual volunteers who abstained from consuming caffeinated foods and beverages to use in their drug cocktail method probing 5 CYP. Otherwise, previous studies have not mentioned probe drug contamination in pooled human plasma and serum commercially available for research.

Our observation emphasizes the importance of testing all lots of blank serum or plasma used in the analytical method for drug contamination.

The calibration curves were linear over the range of 25-12,000 ng/mL for caffeine, 75-36,000 ng/mL for tolbutamide, 0.05-30 ng/mL for dextromethorphan, and 0.1-60 ng/mL for alprazolam with $r^2 > 0.998$ and %Bias <10% at all concentrations (Table 3). Accuracy and precision were assessed over three days (Table 4). The intra-assay and inter-assay %CV and %Bias were <13% (<17% for LLOQ), demonstrating excellent reproducibility. All 4 analytes were stable on the bench-top (room temperature) for 24 h, during 3 freeze/thaw cycles, in the autosampler at 4 °C for 24 h, and during long-term storage for up to 4 months at -80 °C (Table 5). The stock solutions were also stable for up to 4 months.

Matrix effects were investigated by comparing the positive ion electrospray SRM MS/MS response of each analyte spiked into extracts of 6 different lots of human serum to the response obtained for the same analyte in reconstitution solvent [22]. Ideally, the ratio of each pair of responses or “matrix factor” would be unity, whereas ion signal enhancement or suppression would produce a matrix factor >1 or <1, respectively. As indicated in Table 6, no matrix effect was observed for alprazolam. Tolbutamide in human serum extract showed slight ion enhancement, which was corrected by use of an internal standard.

Dextromethorphan in serum extract showed significant ion suppression, which was also corrected through use of an isotopically labeled internal standard (Table 6). Because only 2 out of the 6 lots of human serum used to investigate matrix effects were caffeine-free, the matrix effect for caffeine and the corresponding CV% appear to be large at low concentrations. Based on matrix effect measurements of the internal standard for caffeine, caffeine displayed slight ion enhancement which was consistent across different lots of serum (CV%<7%) (Table 6). At low concentrations corrected for caffeine already present in the serum or at high concentrations, the minor ion enhancement caused by caffeine could be minimized by normalization using an internal standard. Through compensation using isotopically labelled internal standards, minor matrix effects caused by human serum could be overcome and thereby ensure the accuracy of the UHPLC-MS/MS method.

3.3 Method application

This validated UHPLC-MS/MS method was used to analyze serum from 3 Phase I drug-botanical interaction clinical studies each involving 16 subjects, each of whom were administered an oral cocktail consisting of 100 mg caffeine, 250 mg tolbutamide, 30 mg dextromethorphan, and 2 mg alprazolam at baseline and then again after consuming a botanical dietary supplement for 14 days. An example of positive ion electrospray UHPLC-MS/MS SRM chromatograms of all four probe substrates in serum from one subject 3 hours after cocktail administration is shown in Figure 2B. The concentration-time curves for each probe substrate in serum from a subject at baseline and again after intervention with a botanical dietary supplement is shown in Figure 4. The concentrations of caffeine in these serum specimens were determined using the method of standard addition. This UHPLC-MS/MS method was appropriately accurate and sensitive to enable evaluation of the pharmacokinetics of all 4 probe substrates in these drug-botanical dietary supplement interaction studies.

4. Conclusions

A fast, sensitive and specific method was developed and validated for quantitation in human serum of probe substrates from a drug cocktail including caffeine, tolbutamide, dextromethorphan, and alprazolam. This method uses rapid protein precipitation for sample preparation, fast UHPLC separations, and displays excellent peak shape and S/N at the LLOQ. This method is being used to support Phase I clinical studies evaluating whether botanical dietary supplements can cause pharmacokinetic interactions with these CYP substrates. Notably, validation of this method revealed that most commercially available pooled human serum is contaminated with caffeine and alprazolam and, to a lesser extent, dextromethorphan.

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Highlights

- Probe substrate drug cocktail assay was validated for drug interaction studies
- Based on UHPLC-MS/MS, the assay is fast, accurate and sensitive
- Supports pharmacokinetics studies of drug-drug and drug-botanical interactions
- The assay was applied to clinical studies of drug-botanical interactions
- Assay validation revealed probe drugs in blood supply used for research

Figure 1. Structures and fragmentation patterns of the probe substrates used for SRM during UHPLC-MS/MS quantitative analysis including alprazolam (and ring-opened alprazolam), caffeine, dextromethorphan, and tolbutamide.

Figure 2.

Positive ion electrospray UHPLC-MS/MS SRM chromatograms of probe substrates spiked into blank human serum at the LLOQ (A); and levels 3 hours after oral administration of the probe substrate cocktail (B).

Figure 3. Positive ion electrospray UHPLC-MS/MS SRM chromatograms of blank serum from a single individual (A) and pooled human serum sold for research containing alprazolam, caffeine and dextromethorphan (B).

Figure 4. Concentration-time curves in human serum of 4 cytochrome P450 probe substrates following oral administration of a cocktail of 100 mg caffeine, 250 mg tolbutamide, 30 mg dextromethorphan, and 2 mg alprazolam. Each time point represents the mean serum concentration of 2 replicate analyses for a single subject. Overlapping curves represent pre- or post-intervention for 2-weeks with a botanical dietary supplement.

Table 1.

Recoveries of probe substrate analytes from human serum at low, medium and high concentrations (N=3).

Analyte	Concentration (ng/mL)	Recovery
Caffeine	6	98.5%
	500	90.1%
	1000	92.4%
Tolbutamide	18	92.5%
	1500	87.4%
	3000	93.9%
Dextromethorphan	0.15	84.2%
	12.5	87.3%
	25	92.4%
Alprazolam	0.3	90.2%
	25	85.6%
	50	91.2%

Table 2.

Parameters for positive ion electrospray UHPLC-MS/MS analysis of probe substrates and stable isotopically labeled internal standards using collision-induced dissociation and SRM.

Analyte	Retention time (min)	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	Q1 bias	Collision energy (eV)	Q3 bias
Caffeine	1.3	195	138	−14	−22	−14
		195	110	−10	−20	−11
[¹³ C ₃]-Caffeine	1.3	198	140	−13	−22	−14
		198	112	−12	−26	−11
Tolbutamide	2.1	271	172	−10	−19	−17
		271	91	−10	−20	−17
[d ₉]-4-Hydroxy tolbutamide	1.8	296	188	−15	−16	−30
		296	107	−15	−29	−10
Dextromethorphan	1.8	272	215	−11	−26	−14
		272	171	−12	−42	−17
[d ₃]-Dextromethorphan	1.8	275	215	−12	−26	−22
		275	171	−12	−42	−17
Alprazolam	2.0	309	281	−11	−29	−19
		309	205	−11	−42	−22
[d ₅]-Alprazolam	2.0	314	286	−28	−20	−19
		314	210	−13	−57	−21

Table 3.

Linearity, accuracy and precision of calibration standards (N = 3 analytical runs).^a

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9	R ²	Slope
Concentration (ng/mL)											
Caffeine	25	50	100	200	500	1,000	4,000	8,000	12,000		
Mean	2.62	4.93	9.78	212.7	492.0	945.2	3,950	8,001	12,100	0.9996	0.0018
S.D.	0.86	4.2	6.5	17.1	23.5	15.5	38.5	76.7	118.4	0.00015	0.00005
% CV	3.26	8.44	6.69	8.06	4.78	1.64	0.97	0.96	0.98	0.015	2.88
% Bias	4.9	-1.5	-2.2	6.3	-1.6	-5.5	-1.2	0.0	0.8		
Tolbutamide	75	150	300	600	1,500	3,000	12,000	24,000	36,000		
Mean	74.9	140.7	307.8	588.2	1,534	3,033	12,425	24,026	35,494	0.9995	0.0006
S.D.	4.7	6.4	8.0	20.5	58.0	123.3	167.7	189.3	367.1	0.00028	0.00003
% CV	6.25	4.54	2.60	3.49	3.78	4.06	1.35	0.79	1.03	0.03	5.48
% Bias	0.0	-6.2	2.6	-2.0	2.3	1.1	3.5	0.1	-1.4		
Dextromethorphan	0.05	0.1	0.25	0.5	1	5	15	30	N/D ^b		
Mean	0.055	0.105	0.255	0.470	0.955	4.76	14.56	30.76		0.9990	0.0241
S.D.	0.003	0.004	0.002	0.010	0.013	0.005	0.053	0.029		4.4283E-5	3.11395E-5
% CV	4.99	3.24	0.56	2.15	1.34	0.09	0.36	0.09		0.004	0.129
% Bias	10.3	2.8	2.9	-5.8	-4.4	-5.1	-3.0	2.5			
Alprazolam	0.1	0.2	0.5	1	2	10	30	60	N/D		
Mean	0.110	0.210	0.520	0.955	0.933	9.59	29.76	60.85		0.9995	0.0135
S.D.	0.002	0.001	0.003	0.013	0.013	0.046	0.278	0.320		0.0002	0.0002
% CV	1.71	0.36	0.61	1.37	0.68	0.48	0.93	0.53		0.02	1.40
% Bias	10.8	5.1	3.7	-9.4	-6.8	-4.1	-0.8	1.4			

^aWeighting of (1/x) was applied to the curves^bN/D = Not determined (The calibration curves for dextromethorphan and alprazolam contained only 8 concentrations)

Table 4.

Intra-assay and inter-assay accuracy and precision for the quantitative analysis of probe substrates in human serum using UHPLC-MS/MS. (Values are expressed as Mean $\pm$ Standard Deviation)

Probe substrate	Nominal concentration (ng/mL)	Intra-day (N=5)			Inter-day (N=15)		
		Measured (ng/mL)	CV %	Accuracy %	Measured (ng/mL)	CV %	Accuracy %
Caffeine	25	25.65 $\pm$ 1.45	5.66	102.6	25.23 $\pm$ 3.26	12.9	100.9
	60	60.40 $\pm$ 3.26	5.39	100.7	60.14 $\pm$ 5.10	8.49	100.2
	5,000	4714 $\pm$ 151	3.20	94.3	4771 $\pm$ 155	3.3	95.4
	10,000	9678 $\pm$ 199	2.06	96.8	9740 $\pm$ 339	3.5	97.4
Tolbutamide	75	76.79 $\pm$ 5.62	7.33	102.4	81.30 $\pm$ 5.76	7.1	108.4
	180	173.9 $\pm$ 8.0	4.61	96.6	180.2 $\pm$ 110.2	6.1	100.1
	15,000	14807 $\pm$ 930	6.28	98.7	14572 $\pm$ 919	6.3	97.2
	30,000	29776 $\pm$ 1312	4.41	99.3	29568 $\pm$ 1567	5.3	98.6
Dextromethorphan	0.05	0.0583 $\pm$ 0.0024	4.11	116.7	0.0585 $\pm$ 0.0023	4.0	117.0
	0.15	0.156 $\pm$ 0.006	3.98	103.7	0.1500 $\pm$ 0.0069	4.6	100.1
	12.5	12.17 $\pm$ 0.20	1.62	97.4	12.11 $\pm$ 0.36	3.0	96.9
	25	25.24 $\pm$ 0.39	1.54	100.9	25.34 $\pm$ 0.90	3.6	101.4
Alprazolam	0.1	0.110 $\pm$ 0.005	4.7	109.7	0.113 $\pm$ 0.006	5.2	113.0
	0.3	0.288 $\pm$ 0.007	2.4	95.9	0.297 $\pm$ 0.016	5.5	99.0
	25	24.04 $\pm$ 0.36	1.5	96.2	25.09 $\pm$ 1.22	4.8	100.4
	50	48.99 $\pm$ 0.79	1.6	98.0	50.89 $\pm$ 2.22	4.4	101.8

Table 5.

Stability of probe substrates during sample storage and handling. Stock solution stability was determined using 5 replicates. Other stability results were based on measurements of 3 replicate samples at each concentration.

Probe substrate	QC concentration (ng/mL)	Remaining % ($\pm$ S.D.) under different storage condition				
		Freeze/thaw 3 cycles	Autosampler 4 °C, 24 h	Bench top 24 h	Long term -80 °C, 4 months	Stock -20 °C, 4 months
Caffeine	60	97.1 $\pm$ 1.7	103.2 $\pm$ 5.2	97.1 $\pm$ 5.2	105.5 $\pm$ 2.3	98.9 $\pm$ 3.5
	10,000	100.6 $\pm$ 4.6	98.1 $\pm$ 4.8	97.4 $\pm$ 1.7	100.5 $\pm$ 3.1	
Tolbutamide	180	108.0 $\pm$ 2.9	109.5 $\pm$ 0.7	98.0 $\pm$ 7.4	95.8 $\pm$ 9.4	108.4 $\pm$ 3.3
	30,000	107.1 $\pm$ 3.7	98.1 $\pm$ 1.3	98.2 $\pm$ 2.9	94.6 $\pm$ 3.4	
Dextromethorphan	0.15	102.6 $\pm$ 3.8	103.7 $\pm$ 6.6	96.4 $\pm$ 4.7	92.9 $\pm$ 4.5	94.3 $\pm$ 2.1
	50	100.2 $\pm$ 1.0	100.4 $\pm$ 0.8	100.5 $\pm$ 1.6	98.2 $\pm$ 2.6	
Alprazolam	0.3	107.0 $\pm$ 5.4	100.4 $\pm$ 3.0	105.3 $\pm$ 1.8	87.1 $\pm$ 4.5	86.1 $\pm$ 2.8
	100	104.2 $\pm$ 1.1	100.3 $\pm$ 1.9	99.6 $\pm$ 1.3	89.4 $\pm$ 3.0	

Table 6.

Matrix effects of probe substrates and internal standards in 6 lots of blank human serum (N=5). Matrix Factor: $MF_{\text{analyte}} = (\text{Peak area of analyte extracted from serum}) / (\text{Peak area of analyte spiked into reconstitution solvent})$. Internal standard normalized MF = $MF_{\text{analyte}} / MF_{\text{Internal standard}}$

Probe substrate	Concentration (ng/mL)	MF _{analyte}		MF _{Internal standard}		Internal standard normalized MF	
		Mean	CV%	Mean	CV%	Mean	CV%
Caffeine	60	4.45	56.74	1.19	6.88	3.75	53.89
	10,000	1.21	3.00	1.15	3.35	1.06	3.56
Tolbutamide	180	0.99	9.37	1.14	10.55	0.87	5.46
	30,000	1.37	7.44	1.27	6.76	1.08	1.65
Dextromethorphan	0.15	0.43	14.92	0.41	15.71	1.04	3.56
	12.5	0.37	12.08	0.39	13.02	0.95	1.35
Alprazolam	0.3	1.07	3.96	1.09	3.70	0.99	3.00
	50	0.98	1.28	0.97	7.17	1.02	6.69