Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 1241–1249

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Development and validation of a liquid chromatography–tandem mass spectrometry assay for the simultaneous quantitation of prednisolone and dipyridamole in human plasma and its application in a pharmacokinetic study

Mei Chen [a], Camille Granvil [a,1], Qin C. Ji [b], Zhi-Yi Zhang [a], Mahesh V. Padval [a], Vikram V. Kansra [a,*]

[a] Department of Preclinical Development and Formulations, CombinatoRx, Inc., Cambridge, MA 02142, United States
[b] Covance Laboratories Inc., 3301 Kinsman Boulevard, Madison, WI 53704, United States

ARTICLE INFO

Article history:
Received 19 November 2008
Received in revised form 16 February 2009
Accepted 17 February 2009
Available online 25 February 2009

Keywords:
Liquid/liquid extraction
LC–MS/MS
Quantitation
High throughput
Automation
96-Well format

ABSTRACT

We have developed and validated an accurate, sensitive, and robust LC–MS/MS method that determines the concentration of CRx-102 (the combination of prednisolone and dipyridamole) in human plasma. In this method, prednisolone, dipyridamole, and the combined internal standards (IS) prednisolone-d_6 (IS for prednisolone) and dipyridamole-d_{20} (IS for dipyridamole) were extracted from 100 μL human EDTA plasma using methylbutyl ether. Calibration curves were linear over a concentration range of 0.4–200 ng/mL for prednisolone and 5–3000 ng/mL for dipyridamole. The analytes were quantitatively determined using tandem mass spectrometry operated in positive electrospray ionization in a multiple reaction monitoring (MRM) mode. This validated method has been used successfully in clinical pharmacokinetic studies of CRx-102 in healthy volunteers.

1. Introduction

CRx-102 is a synergistic combination drug candidate being evaluated by CombinatoRx, Incorporated, comprising a very low dose of the glucocorticoid prednisolone (3 mg) and the cardiovascular drug dipyridamole (200 mg). The prednisolone contained in CRx-102 is at the low end of the recommended daily maintenance dose that is usually used to treat inflammatory conditions (2.5–15 mg) and is generally considered to be "sub-therapeutic." CRx-102 is thought to act through a novel mechanism of action in which dipyridamole selectively and synergistically enhances the anti-inflammatory and immuno-modulatory effects of prednisolone without amplifying the associated side effects [1]. This co-administration provides a combination science approach to a dissociated glucocorticoid profile. CRx-102 is currently under development for the treatment of rheumatoid arthritis and osteoarthritis. A robust bioanalytical assay that can simultaneously determine the concentrations of both prednisolone and dipyridamole in human plasma is essential in supporting the clinical development of CRx-102 to understand the safety and efficacy of the drug.

Earlier methods developed for the detection for prednisolone have often been based on gas chromatography (GC) [2,3], or GC coupled with mass spectrometry (MS) [4,5], which allows for distinction of prednisolone from endogenous corticosteroids; since the derivatization step prior to GC–MS analysis is cumbersome, it has limited application. Other methods including high-performance liquid chromatography (HPLC) and radioimmunoassay have been widely used for the determination of prednisolone in biological fluids [6–11]. However, these methods do not meet the modern drug discovery and development needs in terms of short run time, high sensitivity, and highly efficient sample preparation procedures. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has become a method of the choice for quantitative analysis of prednisolone concentrations in biological samples [12–16] with a lower limit of quantitation (LLOQ) ranging from 3.6 to 30 ng/mL. With online solid-phase extraction (SPE) coupled with MS [14], a detection limit (LOD) of 10 ng/mL was achieved for prednisolone, and an even lower LLOQ was achieved using a more complicated sample preparation step of protein precipitation (PPT), followed by liquid–liquid extraction (LLE) [16].

Several HPLC methods have been described for the determination of dipyridamole concentration in biological matrix [17–19]. These methods are limited in that they are both relatively insensitive and have a slow turnaround time. Few LC–MS and LC–MS/MS

* Corresponding author at: CombinatoRx, Inc., Department of Preclinical Development and Formulations, 245 First Street, 4th Floor, Cambridge, MA 02142, United States. Tel.: +1 617 301 7084; fax: +1 617 301 7109.
E-mail address: vkansra@combinatorx.com (Vikram V. Kansra).
[1] Current address: Novartis Pharmaceuticals, 180 Park Avenue, Florham Park, NJ 07932, United States.

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.02.017

methods have been published for the determination of dipyridamole concentration in human plasma [20,21] and require the use of high plasma volume (0.5 mL) to achieve a 5 ng/mL or higher of LLOQ. Moreover, none of the methods reported for prednisolone and dipyridamole determination are high-throughput in nature.

The objective of the work described here was to develop and validate a sensitive, simple, and robust high-throughput, 96-well format LLE, LC–MS/MS method that could be used easily for the simultaneous determination of both prednisolone and dipyridamole in human plasma. The LLE sample preparation process is one of the preferred techniques used in assay development because it is both cost-effective and provides higher purity extracts than either SPE or PPT. The steps in the LLE process, including the mixing of sample and extraction solvent, transferring of samples into the 96-well plate, and removal of the extracts to another 96-well plate have typically been performed manually. The introduction of the 96-well format and robotic liquid transfer has significantly improved the throughput of the LLE assays [22–27].

A 96-well LLE extraction method was used for the LC–MS/MS analysis of prednisolone and dipyridamole in human plasma samples. In this method, a liquid handling device (Tomtec, Hamden, CT, USA) was used for the mixing step for all samples and for reagent transfer. The reconstitution step for LC–MS/MS injection may be performed manually or using the liquid handling device. Deuterated prednisolone (prednisolone-d_6) and deuterated dipyridamole (dipyridamole-d_{20}) were used as internal standards (Fig. 1). Since the dose of dipyridamole in CRx-102 (combination of prednisolone + dipyridamole) for clinical studies is much higher than that of prednisolone, differences in the concentrations of each analyte in human plasma samples are expected. Therefore, an extra dilution step was developed to further dilute samples for the analysis of dipyridamole. To the best of our knowledge, studies on LC–MS/MS for the simultaneous determination of prednisolone and dipyridamole in biological samples have not been previously described. The current validated method exhibited higher sensitivity than LC–MS/MS methods previously described for quantitation of prednisolone or dipyridamole alone in a biological matrix. This assay utilizes small sample volume and simple preparation steps. After successful development and validation of this automated method, it was utilized in sample analysis for a clinical pharmacokinetic study of the oral administration of prednisolone alone or CRx-102 in healthy volunteers. The assay demonstrated accuracy, reproducibility, and rigor in a high-throughput analysis of samples in two separate dynamic ranges.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade acetonitrile, HPLC-grade methanol, HPLC-grade concentrated ammonium hydroxide, ammonium acetate, methyl-*tert* butyl ether (MTBE), and ACS grade glacial acetic acid were purchased from Fisher (Pittsburgh, PA, USA). Formic acid (96%) was purchased from Sigma–Aldrich (St. Louis, MO, USA). The reference standards of prednisolone (>99%) and dipyridamole (>98%) were from Sigma–Aldrich (St. Louis, MO, USA). Deuterated internal standards of prednisolone-d_6 and dipyridamole-d_{20} were produced at CDN Isotopes (Pointe Claire, Quebec, Canada) and Covance (Madison, Wisconsin, USA), respectively. Normal human plasma with K_2EDTA as an anticoagulant was purchased from Bioreclamation (Hicksville, NY, USA).

2.2. Instrumentation

The HPLC system utilized a Shimadzu (Kyoto, Japan) LC-10ADvp HPLC pump and a Shimadzu SIL-HTc system controller and autosampler. To deliver backwash solvent for pre-column regeneration, an 1100 series Agilent Technologies (Palo Alto, CA, USA) HPLC pump and degasser system was used. The API 4000 mass spectrometer controlled by Analyst™ software was from Applied Biosystems/MDS Sciex (Concord, ON, Canada). Analyst™ version 1.4 was used as the data acquisition software. The analytical column used was a Genesis C18, 50 mm × 4.6 mm, 3 μm particle size, from Grace Vydac (Chicago, IL, USA). The column heater model CTO-10ASVP was from Shimadzu (Kyoto, Japan). The pre-column inline filter was from Supelco (Atlanta, GA, USA).

2.3. Preparation of standard and QC samples

Standards and QC samples were prepared from two separate stock solutions in parallel. The stock standard solutions of prednisolone (1 mg/mL) [S01] and dipyridamole (1 mg/mL) [S02] were prepared by dissolving each of the accurately weighted reference compounds in methanol. The intermediate standard solutions of 8/120, 10/125, 1/12.5 and 0.1/1.25 μg/mL for prednisolone/dipyridamole were prepared by serial dilutions of stock standard solutions S01 and S02 with methanol:water (50:50, v/v). The stock standard solutions of IS were prepared by dissolving appropriate amounts of deuterated standards in methanol to give a final concentration of prednisolone-d_6 (250 μg/mL) [I01] and dipyridamole-d_{20} (250 μg/mL) [I02]. The intermediate IS solutions of 200 ng/mL of prednisolone-d_6 and 500 ng/mL of dipyridamole-d_{20} [I03] were prepared by adding 40 μL of the prednisolone-d_6 IS stock solution [I01] and 100 μL of the dipyridamole-d_{20} IS stock solution [I02] to a 50 mL glass volumetric flask, then diluting to the volume with methanol:water (50:50, v/v).

The calibration curves with eight non-zero standard levels contain prednisolone/dipyridamole (200/3000, 180/2700, 100/1500, 20/250, 4/50, 2/25, 0.8/10, 0.4/5 mg/mL) in the concentration range of 0.4–200 ng/mL for prednisolone, and 5–3000 ng/mL for dipyridamole. These curves were prepared by adding appropriate volumes of intermediate standard solutions into volumetric flasks and diluting with pooled normal human plasma with K_2EDTA. The QC samples were prepared using the same method at three different concentration levels, the low QC [LQC (1.2/15 ng/mL)], medium QC [MQC (40/500 ng/mL)], and high QC [HQC (150/2250 ng/mL)], for prednisolone and dipyridamole, respectively. The standards and QCs were aliquoted into polypropylene tubes and stored frozen at approximately −60 to −80 °C. Additional QCs were stored at approximately −10 to −30 °C for the purpose of stability evaluation.

2.4. Sample extraction

Samples were thawed at room temperature, protected from light, and vortexed to ensure homogeneity. Each plasma sample tube was manually uncapped and 100 μL were transferred by a manual pipette into the appropriate wells of a 96-well plate. Then, 50 μL of the intermediate IS solution [I03] (200 ng/mL of prednisolone-d_6 and 500 ng/mL of dipyridamole-d_{20}) and 25 μL of concentrated ammonium hydroxide were added to each sample by Eppendorf repeating pipette, followed by a brief vortex to ensure mixing. The liquid handling device was used to add 1000 μL of extraction solvent solution of MTBE to each sample and mix the samples 40 times. Once mixed, 800 μL of the organic phase layer were transferred into an Axygen 96-well collection plate (Plate A). A slow draw rate for the Tomtec program was used. The transfer height with a water/MTBE test plate was tested to ensure no aqueous layer was being transferred before running the program on the actual batch. The wells were dried with SPE Dry-96 at 30 °C under a nitrogen stream and reconstituted with 300 μL of the reconstitution solution of 0.5% acetic acid in 10 mM ammonium acetate:acetonitrile (70:30, v/v) using an Eppendorf

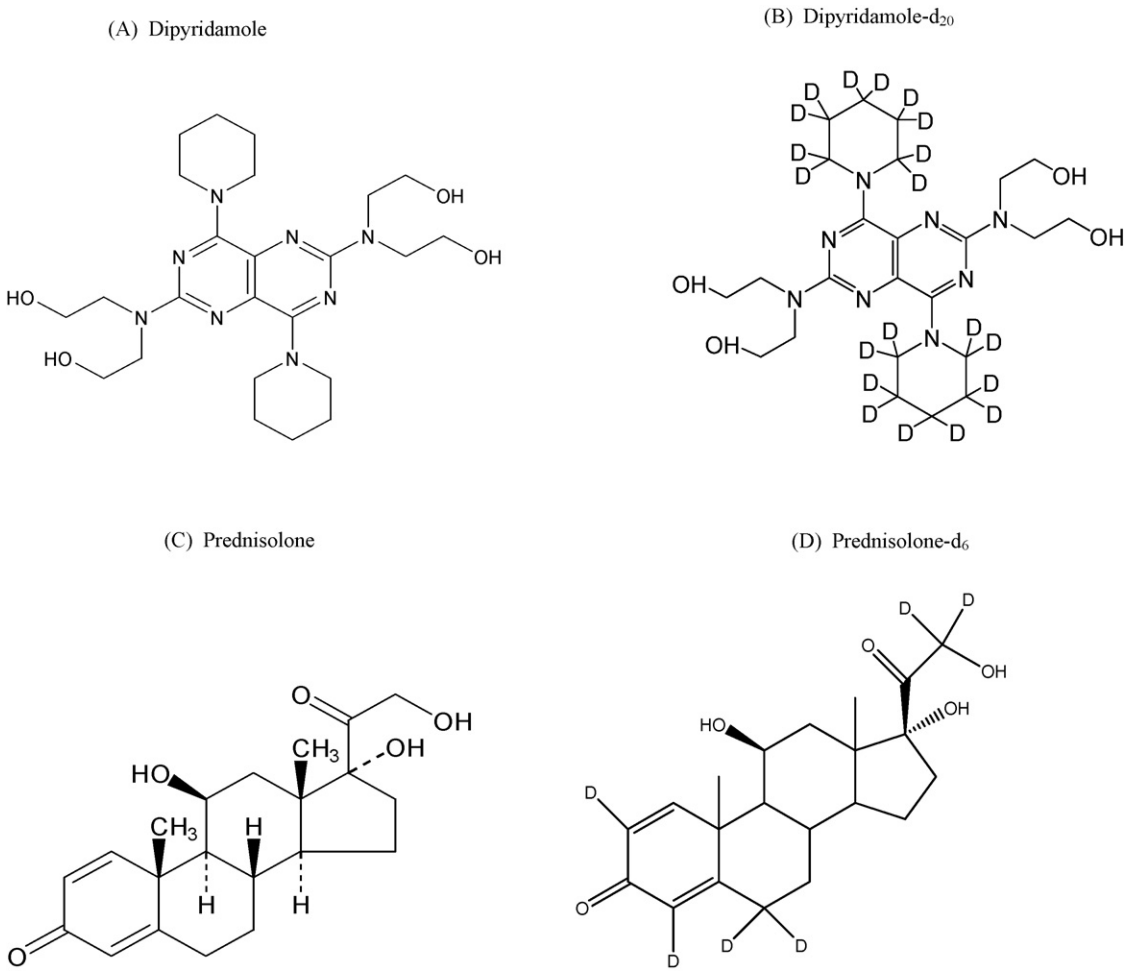

Fig. 1. Structures of (A) dipyridamole, (C) prednisolone, and their internal standards (B) dipyridamole-d_{20} and (D) prednisolone-d_6, respectively.

repeating pipette or liquid handling device. Using the liquid handling device, 25 μL of the reconstituted samples were transferred to an Axygen 96-well collection plate (Plate B) that contained 475 μL of reconstitution solution (0.5% acetic acid in 10 mM ammonium acetate:acetonitrile [70:30, v/v]). The samples were mixed 40 times. From Plate A, 30 μL of the reconstituted samples were injected into the LC–MS/MS system for prednisolone analysis. From Plate B, 10 μL of the reconstituted samples were injected into the LC–MS/MS system for dipyridamole analysis. Since the concentrations of dipyridamole were significantly greater in Plate A than Plate B, Plate B was injected prior to Plate A.

2.5. Chromatographic conditions

A gradient HPLC method was utilized for the separation and the analytical column was kept at 45 °C during the analysis. A flow rate of 1000 μL/min was used. The mobile phase A consisted of 0.1% formic acid in water and the mobile phase B consisted of 0.1% formic acid in acetonitrile. The same gradient was used for the analysis of both analytes.

2.6. MS/MS detection

LC–MS/MS detection was performed using a Sciex API 4000 triple quadrupole mass spectrometer with a Turbo Ionspray® ionization source operated in the positive ion mode. The mass spectrometer was operated under the Analyst™ software version 1.4. The ion spray voltage was 4500 V and the source temperature was 600 °C. The CAD gas setting was 8 and curtain gas setting was 25. The nebulizing gas and auxiliary gas settings were 70 and 65, respectively. Other parameters were optimized by infusing the analytes to the mass spectrometer. The collision energy (CE) differed depending on the ions monitored. For the prednisolone and its internal standard, the CE was 16 and 18 eV, respectively, and for dipyridamole and its internal standard the CE was 85 eV. The selective reaction monitoring (SRM) detection channel for prednisolone was m/z 361 → 325 amu and m/z 367 → 331 amu for prednisolone-d_6 (IS). The SRM detection channel for dipyridamole was m/z 505 → 429 amu and m/z 526 → 449 amu for dipyridamole-d_{20} (IS). The dwell time for each ion monitored was 50 ms.

2.7. Quantitation

The peak areas of both analytes and their ISs were determined using Analyst™ software version 1.4. For each analytical batch, a calibration curve was derived from the peak area ratios (analyte: IS) using weighted linear least squares regression of the area ratio versus the concentration of the standards. A weighting of $1/x^2$ (where x is the concentration of a given standard) was used for curve fitting. The regression equation for the calibration curve was used to back-calculate the measured concentration for each standard and QC. The results were compared to the theoretical concentration to obtain the accuracy, expressed as a percentage of the theoretical value, for each standard and QC measured.

2.8. Pharmacokinetic study in healthy volunteers

In this pharmacokinetic study, 18 healthy subjects received CRx-102 (prednisolone + dipyridamole) in two oral doses, 5 h apart, at 8 a.m. and 1 p.m., respectively. Serial venous blood samples were collected into K_2EDTA tubes before administration and at time points 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 7, 8, 12, 24 and 48 h after administration. Plasma was separated from blood by centrifugation and stored at −20 °C prior to analysis. All pharmacokinetic parameters were estimated by using Pharsight WinNonLin 4.0 software.

3. Results and discussion

3.1. Sample extraction

PPT was initially developed with methanol and acetonitrile, but this technique resulted in strong interferences from the sample matrix. LLE was developed because it cleans and concentrates the samples. Analytes that were acidic or basic required adjustment to neutral pH prior to extraction. Dipyridamole, as a basic analyte with multiple amine groups, required the addition of base to the aqueous sample to promote equilibrium to the unionized form for extraction. Before extraction, 25 μL of concentrated ammonium hydroxide was used to adjust the pH of dipyridamole at least two units greater than the pK_a of the analyte. As prednisolone is a neutral analyte not affected by pH adjustment, it was extracted with dipyridamole into the organic phase. The standards, QCs, and plasma samples were manually transferred from the polypropylene tubes to a 2.2 mL 96-well plate. ISs and buffer were added and mixed, followed by the addition and mixing of the organic extraction solvent. In order to have an assay concentration range compatible with clinical samples, the LLOQ value for prednisolone was 0.4 ng/mL, and 5 ng/mL for dipyridamole. The reconstituted solution from Plate A was diluted 20 times further to create Plate B for the analysis of dipyridamole. Plates A and B were injected separately to accommodate the difference in curve ranges and instrument responses between prednisolone and dipyridamole. The liquid handling device was used for aspirating and dispensing during the organic extraction step.

3.2. LC–MS/MS detection

MS and tandem MS were obtained by the infusion of dipyridamole and prednisolone solutions via a tee connection between the LC column and mass spectrometer inlet. The protonated peak m/z 361 is the main molecular ion of prednisolone. The MS/MS spectra of prednisolone give two major product ions at m/z 343 and 325. Although the product ion of 325 has less intensity compared to 343, the final ion precursor → product ion combination of m/z 361 → 325 was chosen for detection of prednisolone since there is low background effect from the matrix under this condition. There are eight nitrogen atoms in the dipyridamole structure that are potentially good sites for protonation. However, the full scan ES mass spectrum is composed of only one major peak at m/z 505 for $[M+H]^+$, which may be caused by the first protonation drastically decreasing the basicity of all nitrogen atoms [28]. Isotopically labeled ISs for both prednisolone and dipyridamole give molecular ions and product ions at 20 and 6 mass-to-charge units difference, respectively (spectra not shown). Although there are significant differences in chemical properties between dipyridamole and prednisolone, the chromatographic method was developed to have adequate retention for both analytes. As shown in Fig. 2, the retention time is approximately 1.7 min for prednisolone and 1.4 min for dipyridamole, with retention times of 1.7 and 1.4 min for the ISs of prednisolone and dipyridamole, respectively.

3.3. Assay validation

Validation experiments were designed with reference to the Guidance for Industry-Bioanalytical Method Validation recommended by the Food and Drug Administration (FDA) of the United States [25]. The experimental design and results of important criteria of method validation are presented in the following sections.

3.4. Linearity, LLOQ, and ULOQ, dilution

The linearity of the calibration curve was evaluated from three consecutively prepared batches. The linear dynamic range was between 0.4 and 200 ng/mL for prednisolone and between 5 and 3000 ng/mL for dipyridamole. The calibration curve coefficient of determination (r^2) was at least 0.99 for both prednisolone and dipyridamole. The mean back-calculated concentrations of the standards were between 92.8% and 106% of the theoretical concentrations (Table 1) for prednisolone and between 94.7% and 104.8% of theoretical for dipyridamole.

Twelve replicates of LLOQ samples were used to evaluate the precision and accuracy at the low end of the assay range from three separate runs. For prednisolone, the coefficient of variation (CV) was 9.9% and the accuracy, expressed as percent theoretical, was 105%. For dipyridamole, the CV was 8.3% and the accuracy was 102.6%. Representative LC–MS/MS chromatograms of LLOQ samples are shown in Fig. 2.

The suitability of study samples being diluted with drug-free plasma on the day of assay without undergoing an additional freeze–thaw cycle was evaluated as part of the validation. A QC level used specifically for dilutions was prepared with the concentrations of both analytes above the ULOQs during validation at 600 ng/mL for prednisolone and 10,000 ng/mL for dipyridamole. To achieve a 10-fold dilution, 10 μL of dilution QC and 90 μL of blank matrix were combined in the 96-well plate and extracted as normal. Results for dilution QCs gave a mean accuracy of 94.2% for prednisolone and 104% for dipyridamole. Corresponding CVs were 3.2% and 1.6% for prednisolone and dipyridamole, respectively.

3.5. Precision and accuracy

Eighteen replicates of QC samples from three consecutive runs were used to evaluate precision and accuracy at each concentration level. For prednisolone QC at concentrations of 1.2, 40, and 150 ng/mL, the intra-assay CV was between 1.3% and 4.9%, and the inter-assay CV was between 2.8% and 5.4%. The inter-assay mean accuracies, expressed as percents of theoretical, were between 97.3% and 106.3%. For dipyridamole QC at concentrations of 15, 500, and 2250 ng/mL, the intra-assay CV was between 0.8% and 3.6%, and the inter-assay CV was between 1.8% and 3.6%. The inter-assay mean accuracies, expressed as percents of theoretical, were between 98.7% and 106.2% (Table 2).

3.6. Selectivity

Selectivity was evaluated by extracting blank human K_2EDTA plasma from six different lots of matrix and comparing the MS/MS response at the retention times of prednisolone and dipyridamole to the responses of the LLOQ. In five of six cases, the prednisolone, dipyridamole, prednisolone-d_6, and dipyridamole-d_{20} regions were free from significant interference, which means that the peak areas were <20% of the mean utilized LLOQs or <5% of IS response in the control zero sample. No significant peaks were observed in any of the blank plasma samples for either prednisolone or dipyridamole.

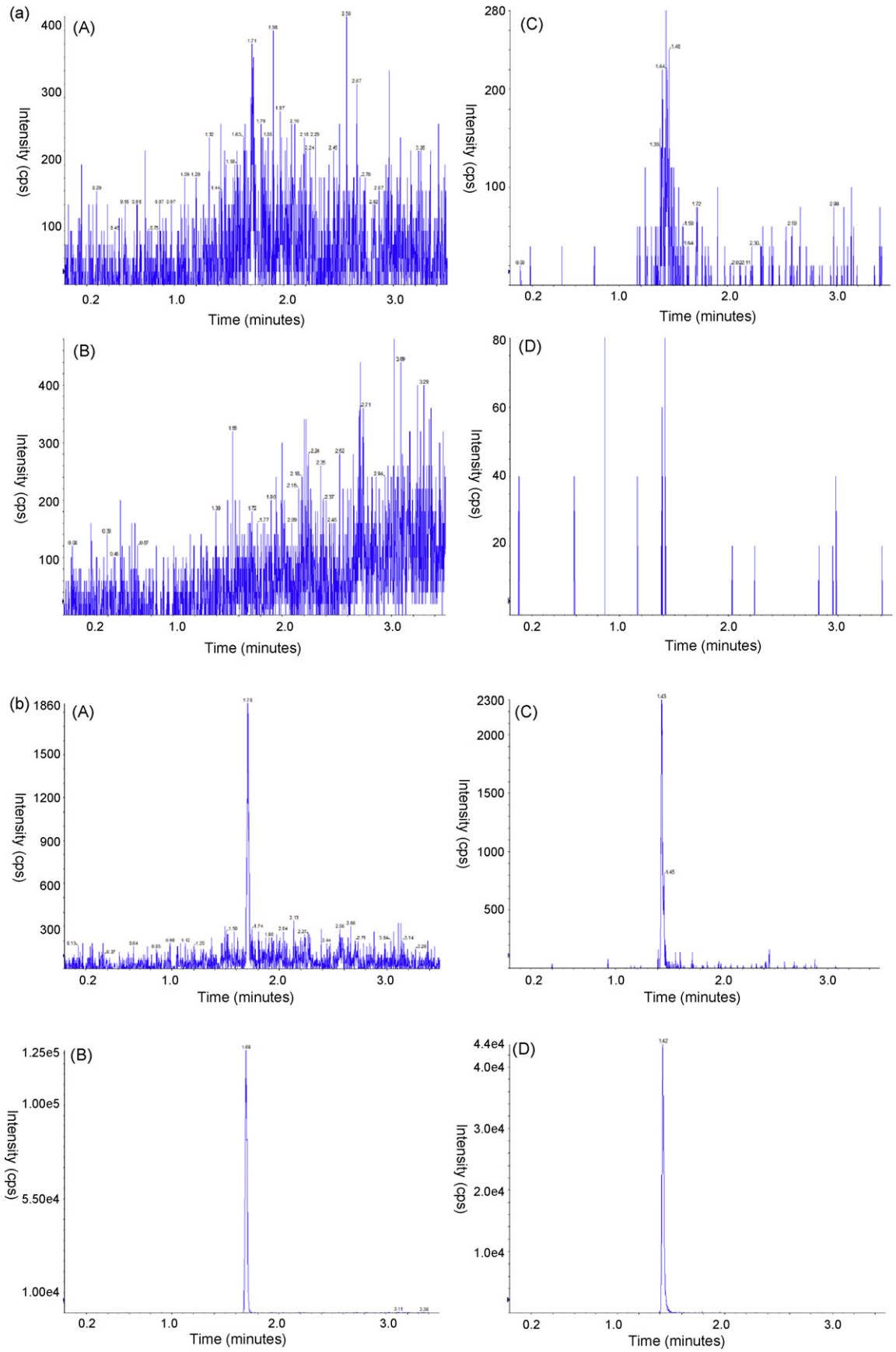

Fig. 2. (a) Representative chromatograms of: prednisolone (A), prednisolone-d_6 (B), dipyridamole (C) and dipyridamole-d_{20} (D) in human blank plasma. (b) Representative chromatograms of LLOQ of 0.4 ng/mL prednisolone (A) in human plasma and 50 ng/mL dipyridamole (C) in human plasma, with prednisolone-d_6 (B) and dipyridamole-d_{20} (D) as IS for prednisolone and dipyridamole, respectively.

Table 1
Statistical calculation of human plasma calibration standards for assay linearity validation for the analyte.

Analysis	Theoretical concentration (ng/mL) (n = 3)							
Group	0.4	0.8	2.0	4.0	20.0	100.0	180.0	200.0
Prednisolone								
003	0.4	0.8	2.1	4.3	20.1	104.0	169.0	186.0
004	0.4	0.8	2.0	4.2	21.4	105.0	168.0	183.0
005	0.4	0.8	2.2	4.2	20.0	102.0	163.0	189.0
Mean	0.4	0.8	2.1	4.2	20.5	104.0	167.0	186.0
S.D.	0.008	0.03	0.1	0.1	0.8	1.5	3.2	3.0
R.S.D. (%)	2.1	3.7	4.7	1.4	3.8	1.5	1.9	1.6
Accuracy (%)	98.8	99.8	103.5	106.0	102.5	104.0	92.8	93.0
Analysis	Theoretical concentration (ng/mL) (n = 3)							
Group	5.0	10.0	25.0	50.0	250.0	1500.0	2700.0	3000.0
Dipyridamole								
003	4.9	10.2	25.2	51.4	265.0	1510.0	2540.0	2870.0
004	5.0	10.1	24.7	52.8	262.0	1560.0	2560.0	2760.0
005	4.9	10.4	24.1	52.7	259.0	1500.0	2600.0	2900.0
Mean	4.9	10.2	24.7	52.3	262.0	1520.0	2570.0	2840.0
S.D.	0.02	0.2	0.6	0.8	3.0	32.1	30.6	73.7
R.S.D. (%)	0.4	1.5	2.2	1.5	1.1	2.1	1.2	2.6
Accuracy (%)	98.8	102.0	98.8	104.6	104.8	101.3	95.2	94.7

3.7. Matrix effect

The effect of the plasma matrix on concentration determination was determined by preparing QC samples spiked in human K_2EDTA plasma with six different individual lots of matrix, using the following equation:

$$\text{matrix effect}\,(\%) = \frac{(\text{mean post extraction peak area} - \text{mean pure solution peak area})}{(\text{mean pure solution peak area})} \times 100$$

positive value indicates percent enhancement, negative value indicates percent suppression.

The concentrations of the QCs were evaluated using a calibration curve generated from the same standards used for the determination of linearity, precision, and accuracy. Quantitated by this curve, the matrix effects for prednisolone at 40 ng/mL and dipyridamole at 500 ng/mL were between 3.1–5.2% and 1.2–3.2%, respectively, demonstrating that the measured concentrations of both the analyte and metabolite are independent of the sample matrix (Table 3).

3.8. Extraction recovery

In order to determine extraction recovery, six replicates of the mean peak area of recovery control samples, at three concentration levels (LQC, MQC, and HQC) for each analyte, were compared to six replicates of mean peak area of the LQC, MQC, and HQC plasma samples post-extraction. The recovery control samples were prepared in the reconstitution solvent at the known concentrations. The plasma samples were extracted with IS and, after drying, were reconstituted as normal. The peak area ratio of analyte/IS for the recovery controls (RC) and extracted QC samples were determined. Extraction recovery was calculated by dividing the area ratios of individual extracted QCs plasma samples by the mean area ratio of the recovery control QCs samples at the corresponding concen-

Table 2
Statistical calculation of human plasma QC samples for assay accuracy and precision validation.

Group	Analysis	Theoretical concentration (ng/mL)					
		Prednisolone (n = 6)			Dipyridamole (n = 6)		
		1.2	40.0	150.0	15.0	500.0	2250.0
1	Within-group mean	1.3	42.2	148.0	15.2	540.0	2240.0
	S.D.	0.1	1.6	5.2	0.5	4.3	39.4
	R.S.D. (%)	4.9	3.7	3.5	3.6	0.8	1.8
	Accuracy (%)	104.2	105.5	98.7	101.3	108.0	99.6
2	Within-group mean	1.2	42.0	145.0	15.5	533.0	2210.0
	SD	0.04	0.9	4.4	0.5	9.2	31.0
	RSD (%)	3.2	2.2	3.0	3.1	1.7	1.4
	Accuracy (%)	100.8	105.0	96.7	103.3	106.6	98.2
3	Within-group mean	1.3	43.5	146.0	14.9	521.0	2220.0
	S.D.	0.05	0.6	2.4	0.5	11.0	48.9
	R.S.D. (%)	3.9	1.3	1.7	3.1	2.1	2.2
	Accuracy (%)	110.0	108.8	97.3	99.3	104.2	98.7
	Overall mean (n = 18)	1.3	42.5	146.0	15.2	531.0	2220.0
	S.D.	0.07	1.23	4.1	0.5	11.6	39.5
	R.S.D. (%)	5.4	2.9	2.8	3.6	2.2	1.8
	Accuracy (%)	105.0	106.3	97.3	101.3	106.2	98.7

Table 3
Summary of matrix effect QC sample evaluation.

Analysis	Theoretical concentration (ng/mL)	Prednisolone		IS	
Group		Post-extraction spike Peak area	Pure solution Peak area	Post-extraction spike Peak area	Pure solution Peak area
005	40.0				
n		6	3	6	3
Mean		174503.8	165934.1	174392.3	169192.6
S.D.		8399.8	1664.4	5078.4	1262.7
R.S.D. (%)		4.8	1.0	2.9	0.7
Matrix effect (%)		5.2		3.1	
Analysis	Theoretical concentration (ng/mL)	Dipyridamole		IS	
Group		Post-extraction spike Peak area	Pure solution Peak area	Post-extraction spike Peak area	Pure solution Peak area
005	500.0				
n		6	3	6	3
Mean		289512.8	286096.7	53342.3	51680.8
S.D.		11598.0	7890.7	2789.0	822.4
R.S.D. (%)		4.0	2.8	5.2	1.6
Matrix effect (%)		1.2		3.2	

tration. Evaluated at prednisolone concentration levels of 1.2, 40, and 150 ng/mL, the mean extraction recoveries were in the range of 97–104.3% with %CV ≤4.8%, and the extraction recovery of its IS was 99.1%. Evaluated at dipyridamole concentration levels of 15, 500 and 2250 ng/mL, the mean extraction recoveries were in the range of 92.2–104% with %CV ≤3.8%, and the extraction recovery of its IS was 100.7%. These results demonstrate that the extraction recovery is adequate to achieve accurate, precise, and reproducible results at the LLOQ.

3.9. Stability

Stability of standard stock solutions of prednisolone, dipyridamole, prednisolone-d_6, and dipyridamole-d_{20} in methanol used in the preparation of standards and QCs was established at both ambient temperatures and at −10 to −30 °C as part of the validation. Results for the determination of stock solution stability were calculated by comparing mean response ratios (area of response per unit of concentration) of stability solutions to mean response ratios of freshly prepared control solutions. Room temperature stability of all four stock solutions was established for at least 6 h. In addition, the stability of stock standard solutions of prednisolone and dipyridamole was tested by comparing freshly prepared stock standard solutions with solutions that were stored in a freezer set to maintain between −10 and −30 °C for 69 days. Results showed that the difference between the stored solutions was ≤5%, thereby indicating acceptable stability for these durations of storage.

The stability of samples subjected to three freeze/thaw cycles with a corresponding storage period at room temperature was studied at two concentration levels (low and high QC) in six replicates (Table 4). Frozen storage stability of prednisolone and dipyridamole in matrix was evaluated by preparing six sets of stability QC at three concentration levels (low, medium, and high QC) and storing them at −60 to −80 °C. The mean concentration of each QC level was compared to each mean concentration determined in the initial testing. The stability of processed samples in reconstitution solution was determined by extracting a calibration curve and six replicates of low, medium, and high QC samples, which were processed and stored refrigerated at 2–8 °C for 95 h (prednisolone) or 91 h (dipyridamole) before analysis by LC–MS/MS. The results show that each analyte had an acceptable stability under the test conditions (Table 4).

3.10. Assay application for clinical studies

The assay described here was applied to a clinical pharmacokinetics study in 18 human subjects after daily administration of CRx-102 (prednisolone + dipyridamole) at 8 a.m. and 1 p.m. The Day 1 mean plasma concentration–time profiles of prednisolone and dipyridamole are shown in Fig. 3. The concentration–time data were analyzed by non-compartmental method using WinNonlin (4.0). Following the dose of prednisolone as part of CRx-102, the maximum mean plasma concentration (C_{max}) was 85.1 ng/mL (%CV = 23.9), and the area under the plasma concentration–time curve from 0–24 h ($AUC_{0-24\,h}$) was 491 ng/mL × h (%CV = 15.1). The time to reach C_{max} (T_{max}) was in a range between 0.5 and 2 h with a median T_{max} of 0.8 h. The elimination half-life was 2.6 h (%CV = 12.5) for prednisolone as part of CRx-102. The apparent systemic clearance (CL/F) was 101 mL/min (%CV = 15.8), and the apparent volume of distribution (V_d/F) was 22.7 L (%CV = 11.9) for prednisolone as part of CRx-102. The pharmacokinetic parameters obtained in this study were comparable with previous data reported for prednisolone administered as a single agent [29,30].

Following the dose of dipyridamole as part of CRx-102, it was rapidly absorbed from the gut, with a T_{max} range between 0.5 and 6 h and a median T_{max} of 3.5 h. The C_{max} was 2081 ng/mL (%CV = 35.8) and the $AUC_{0-24\,h}$ was 11,864 ng/mL × h (%CV = 31.5).

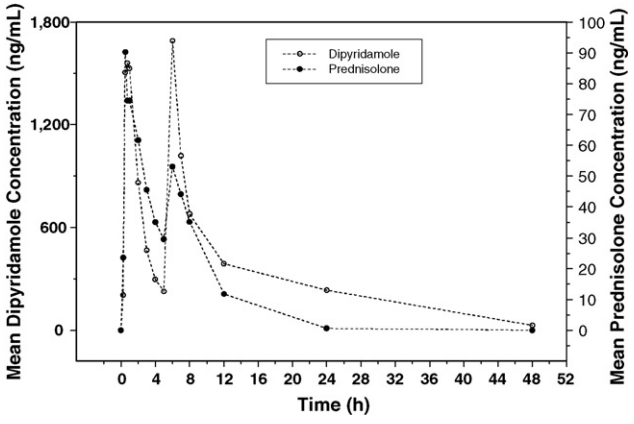

Fig. 3. Mean plasma concentration curves of prednisolone and dipyridamole over time in 18 healthy subjects administered CRx-102 (prednisolone + dipyridamole).

Table 4
Stability of prednisolone and dipyridamole in human plasma ($n = 6$).

Drug	Theoretical concentration (ng/mL)	Sample condition									
		Bench top stability[a]		Freeze–thaw stability[b]		Processed-sample stability[c]		Frozen matrix stability (104 days)[d]		Frozen matrix stability (174 days)[d]	
		Accuracy (%)	R.S.D. (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)	R.S.D. (%)
Prednisolone	1.2	102.5	6.1	104.2	2.9	105.8	6.9	103.3	7.1	100.8	9.9
	40.0	–	–	–	–	110.5	4.8	99.3	3.3	98.8	3.5
	150.0	96.7	2.7	98.0	4.4	98.7	3.1	98.0	2.3	94.7	4.1
Dipyridamole	15.0	100.0	2.3	100.0	3.1	102.7	2.7	94.7	2.7	88.0	2.1
	500.0	–	–	–	–	108.0	2.1	93.6	3.3	103.4	3.0
	2250.0	100.4	1.1	98.2	1.1	99.6	1.3	95.6	2.3	99.1	3.0

[a] Room temperature for 24 h.
[b] Freeze–thaw in three cycles.
[c] Refrigerated (2–8 °C) for 95 h.
[d] Stored at −60 to −80 °C.

The elimination half-life was 8.5 h (%CV = 22.1) for dipyridamole as part of CRx-102. The apparent systemic clearance (CL/F) was 240 ± mL/min (%CV = 37.1) and V_d/F was 177 L (%CV = 32.2) for dipyridamole as part of CRx-102. The pharmacokinetic parameters obtained in this study were generally comparable with previous data reported for dipyridamole [31–32].

4. Conclusions

The high-throughput 96-well format LLE LC–MS/MS method was effective for the simultaneous quantitative analysis of prednisolone and dipyridamole in human plasma. The utilization of a liquid handling device simplified the extraction procedure, minimized the time of sample preparation, and reduced the likelihood of human error. The analytical method was proven to be consistent and reproducible for both analytes from human plasma with minimum interference and short chromatographic run time (3.5 min). The method is sensitive, with a LLOQ of 0.4 ng/mL for prednisolone and 5 ng/mL for dipyridamole, using a small sample volume of 100 μL. The success of the validated method allowed for its application in a pharmacokinetic study, providing efficient and timely support of further clinical studies.

Acknowledgments

The authors wish to thank Ryan Hurd for coordinating the bioanalytical study at Covance. We would also like to thank John Newton and Karen D'Amour for their assistance in the preparation of this manuscript.

References

[1] G.R. Zimmermann, W. Avery, A.L. Finelli, M. Farwell, C.C. Fraser, A.A. Borisy, Arthritis Res. Ther. 11 (2009) R12.
[2] G.E. Bacon, S. Kokenakes, Lab. Clin. Med. 73 (1969) 1030–1035.
[3] D. Murphy, H.F. West, J. Clin. Pathol. 21 (1968) 372–375.
[4] G.M. Rodchenkov, A.N. Vedenin, V.P. Uralets, V.A. Semenov, J. Chromatogr. 565 (1968) 45–51.
[5] H. Shibasaki, T. Furuta, Y. Kasuya, J. Chromatogr. 579 (1992) 193–202.
[6] R. Oertel, W. Kirch, E. Klemm, Pharmazie 62 (2007) 239–240.
[7] Y. Zhang, H.L. Wu, Y.J. Ding, A.L. Xia, H. Cui, R.Q. Yu, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 840 (2006) 116–123.
[8] G. Suarez-Kurtz, C. Estrela Rde, M.C. Salvadori, Ther. Drug Monit. 26 (2004) 16–22.
[9] T. Tobita, M. Senarita, A. Hara, J. Kusakari, Hear. Res. 165 (2002) 30–34.
[10] B.J. Koopman, J.C. van der Molen, E.B. Haagsma, et al., J. Clin. Chem. Clin. Biochem. 24 (1986) 831–839.
[11] L. Bastholt, C.J. Johansson, P. Pfeiffer, et al., Cancer Chemother. Pharmacol. 28 (1991) 205–210.
[12] R. Difrancesco, V. Frerichs, J. Donnelly, C. Hagler, J. Hochreiter, K.M. Tornatore, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 859 (2007) 42–51.
[13] V.A. Frerichs, K.M. Tornatore, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 802 (2004) 329–338.
[14] L.J. Owen, S. Gillingwater, B.G. Keevil, Ann. Clin. Biochem. 42 (2005) 105–111.
[15] M.W. van Hout, C.M. Hofland, H.A. Niederländer, A.P. Bruins, R.A. de Zeeuw, G.J. de Jong, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 794 (2003) 185–192.
[16] R.L. Taylor, S.K. Grebe, R.J. Singh, Clin. Chem. 50 (2004) 2345–2352, epub 2004 October 14.
[17] M. Barberi, J.L. Merlin, B. Weber, J. Chromatogr. 565 (1991) 511–515.
[18] C. Ji, W. Na, X. Fei, C. Sheng-Jun, Z. Jia-Bi, J. Drug Target 14 (2006) 717–724.
[19] J. Cheng, J.B. Zhu, N. Wen, F. Xiong, Int. J. Pharm. 313 (2006) 136–143, epub 2006 March 15.
[20] M. Yadav, D. Patel, P. Singhal, R. Prasad, S. Goswami, P.S. Shrivastav, U.C. Pande, Rapid Commun. Mass Spectrom. 22 (2008) 511–518.
[21] N. Wang, F. Xu, Z. Zhang, C. Yang, X. Sun, J. Li, Biomed. Chromatogr. 22 (2008) 149–156.
[22] S. Steinborner, J. Henion, Anal. Chem. 71 (1999) 2340–2345.
[23] M. Jemal, D. Teitz, Z. Ouyang, S. Khan, J. Chromatogr. B Biomed. Sci. Appl. 732 (1999) 501–508.
[24] N. Zhang, K.L. Hoffman, W. Li, D.T. Rossi, J. Pharm. Biomed. Anal. 22 (2000) 131–138.
[25] J. Zweigenbaum, J. Henion, Anal. Chem. 72 (2000) 2446–2454.

[26] L. Ramos, R. Bakhtiar, F.L.S. Tse, Rapid Commun. Mass Spectrom. 14 (2000) 740–745.
[27] S.H. Hoke 2nd, J.A. Tomlinson 2nd, R.D. Bolden, K.L. Morand, J.D. Pinkston, K.R. Wehmeyer, Anal. Chem. 73 (2001) 3083–3088.
[28] E. Rodrigues Filho, A.M. Paixão Almeida, M. Tabak, J. Mass Spectrom. 38 (2003) 540–547.
[29] S. Rohatagi, J. Barth, H. Mollmann, J. Clin. Pharmacol. 37 (1997) 916–925.
[30] A. Taggart, C. Astbury, J. Dixon, H. Bird, Clin. Rheumatol. 5 (1986) 327–331.
[31] C. Mahony, K. Wolfram, D. Cocchetto, T. Bjornsson, Clin. Pharmacol. Ther. 31 (1982) 330–338.
[32] T. Bjornsson, C. Mahony, Thromb. Res. Suppl. 4 (1983) 93–104.