Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 5, Suppl 3, 2013

Research Article

SIMULTANEOUS DETERMINATION OF DAPSONE AND ITS MAJOR METABOLITE N-ACETYL DAPSONE BY LC-MS/MS METHOD

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Received: 03 Jun 2013, Revised and Accepted: 24 Jun 2013

ABSTRACT

Objective: To develop a rapid, simple and sensitive isocratic reversed-phase high performance liquid chromatographic/Tandem mass spectrometeric (LC/MS-MS) method for simultaneous determination of Dapsone and N-Acetyl Dapsone in human plasma using Dapsone D8 as internal standard (IS).

Methods: The plasma samples were subjected to Liquid-liquid extraction using tertiary butyl methyl ether.Seperation was achieved on a Chromolith C18 Hi-resolution column (100mm×4.6mm ID) using Acetonitrile and 2mM Ammonium acetate as a mobile phase by isocratic elution at a flow rate of 0.8 mL/min. Detection was performed using electrospray ionization in positive ion multiple reaction monitoring (MRM) mode by monitoring the ion transitions from m/z 249.3→156.1 (Dapsone), m/z 291.1→156.0 (N-Acetyl Dapsone) and m/z 257.3→160.0 (IS).

Results: Calibration curves were linear in the concentration range of 0.50–2,500.00 ng/mL for Dapsone, and 0.25–20.00 ng/mL for N-Acetyl Dapsone. The method was validated for selectivity, sensitivity, recovery, linearity, accuracy and precision and stability studies. Recoveries obtained were consistent and reproducible. The intra- and inter-run precisions were less than 15% and the accuracies were within 100±15% for both Dapsone and N-Acetyl Dapsone.

Conclusion: The method has been validated for the simultaneous determination of both Dapsone and N-Acetyl Dapsone in Human plasma and can be applied to pharmacokinetic studies of Dapsone and N-Acetyl Dapsone including the determination of phenotype acetylators.

Keywords: Dapsone, N-Acetyl Dapsone, LC-MS/MS, Validated method.

INTRODUCTION

Dapsone (Fig 1.) is widely used for a number of disorders. It is the drug of choice for leprosy, being one of the components of the multidrug regimen advised by the World Health Organization.[1-2] Other therapeutic uses include the treatment of chloroquine-resistant malaria,[3] brown recluse spider bites,[4] and certain chronic disorders with an autoimmune component.[5-7] More recently it has been advocated and used extensively as an alternative therapy for the treatment of Kaposi's sarcoma[8] and Pneumocystis carinii pneumonia in patients with acquired immunodeficiency syndrome.[9-10] As an antibacterial, dapsone inhibits bacterial synthesis of dihydrofolic acid, via competition with para-aminobenzoate for the active site of dihydropteroate synthetase. Though structurally distinct from dapsone, the sulfonamide group of antibacterial drugs also works in this way. Dapsone is almost completely absorbed from the GI tract with peak plasma concentrations occurring about 2-8 hours after a dose.[11] Steady-state concentrations are not obtained until after at least 8 days of daily administration; doses of 100mg daily provide trough concentrations of 0.5 micrograms/ml. About 50-80% of dapsone in the circulation is bound to plasma proteins and nearly 100% of its monoacetylated metabolite is bound; Dapsone undergoes enterohepatic recycling. It is widely distributed; is present in saliva, breast milk and crosses the placenta. The half-life ranges from 10-80 hours. Dapsone is acetylated to monoacetyldapsone, the major metabolite, and other mono and diacetyl derivatives. [12], Acetylation exhibits genetic polymorphism. [13-14] Hydroxylation is the other major metabolite pathway resulting in hydroxylamine dapsone which may be responsible for dapsone-associated methaemoglobinaemia and haemolysis. Dapsone is mainly excreted in the urine, only 20% of a dose as unchanged drug. Dapsone is administered orally as a 100 mg tablet or alternatively as 25 mg tablets. To deal with dapsone-resistant leprosy cases, multidrug therapy was introduced by WHO in 1981;dapsone is administered along with rifampin and clofazimine or other antileprotic drugs. Dapsone is administered transdermally (via the skin) as a gel 5% topical acne medication and available in 3-, 30-, and 60-gram tubes. In normal use, 0.5 grams should be administered to the face per application twice a day.

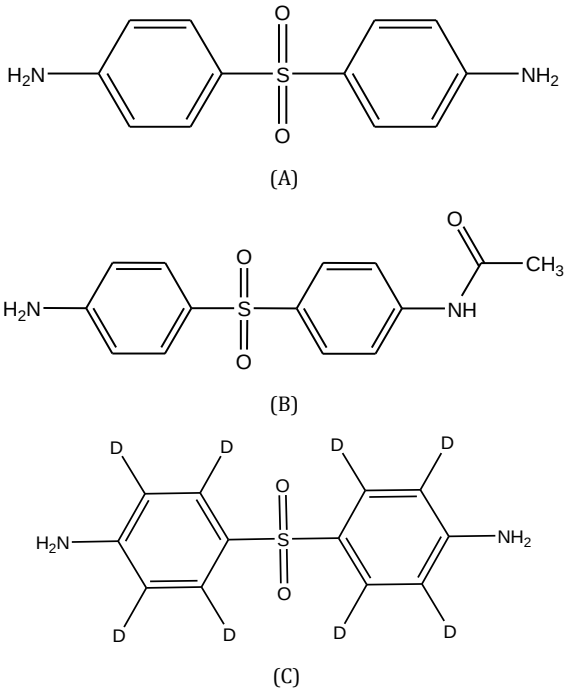

Fig. 1: chemical structure of (A) Dapsone, (B) N-Acetyl Dapsone and (C) Dapsone D8

Since Dapsone is administered in various dosage forms and in different strengths, an analytical method with wide calibration range is required and which we have achieved in the present method. Literature survey reveals that only few methods are reported to determine Dapsone in variety of matrices like rat plasma and human plasma. Few analytical methods for determination of Dapsone in human plasma have been reported, including liquid chromatography coupled to UV detector [15] and liquid chromatography–mass spectrometric (HPLC–MS)[16]. However, only one method is reported till date for simultaneous determination of Dapsone and N-

Acetyl Dapsone in human plasma [17], but the calibration curve range of Dapsone doesn't cover the Cmax range on the higher concentration. In this study, we report a simple, sensitive and specific LC–MS/MS assay for simultaneous quantification of both the drugs in human plasma and it was suitable for pharmacokinetic study covering the Cmax range of Dapsone.

In the present investigation, we have developed a method having a shorter run time with simple liquid-liquid extraction technique. The following are the advantages of the proposed method over those reported earlier: (i) Sample to be collected for time point from individual during the study was reduced significantly. This allows inclusion of additional time points for sample collection;(ii) Employing a single step liquid-liquid extraction method simplified the sample extraction procedure ,minimizes the chances of errors and saves considerable time;(iii) The use of deuterated internal standard, which is physically and chemically identical to the Analyte thus minimizing the errors during sample preparation and mass spectrometer detection. The above points, low plasma volume, use of deuterated internal standard, liquid-liquid extraction and a run time of 2.5 Min. makes the method an attractive procedure in high throughput bioanalysis of Dapsone in human plasma.

MATERIALS AND METHOD

Chemicals and reagents

Dapsone and Dapsone D8 was procured from clearsynth labs pvt. ltd., N-Acetyl Dapsone was synthesized form Vergo pharma. Acetonitrile of HPLC grade was obtained from J.T.Baker. Water was deionized and purified by using Milli-Q system (Millipore, Milford, MA, USA). Tertiary butyl methyl ether and ammonium acetate obtained from Merck Company (Darmstadt, Germany).

Instrumentation and chromatographic conditions

Shimadzu HPLC System consisting of LC-20AD Prominence LIQUID CHROMATOGRAPH Pump, SIL-HTc Auto sampler, andShimadzu CTO-10AS vp Column oven was used for chromatography. The separation was performed on a Chromolith C18 Hi-resolution column (100mm×4.6mm ID) with an isocratic elution. The mobile phase consisted of Acetonitrile:2mM ammonium acetate in water (90:10, v/v).The flow rate was 0.8 mL/min. The HPLC system was coupled to an API 4000 triple–quadrupole mass spectrometer (AB Sciex, Foster City, CA, USA) equipped with an electrospray ionization (ESI) source. The turbo ion-spray interface was operated in both positive-ion mode with nitrogen as the curtain gas, CAD gas, GS1 and GS2 with the optimum values set at 20,4,40 and 50 respectively. The Source temperature was set at 500 ◦ C and the ESI needle voltages in positive-ion mode was adjusted to 5500 V. Quadrupoles Q1 and Q3 were set to unit resolution. The unit dwell time is 200ms. Analytes were quantificated by multiple reaction monitoring (MRM) employing the following precursor to product ion transitions and the parameters are: Dapsone, m/z 249.300→156.100 with declustering potential (DP) 75V and collision energy (CE) 23 eV; Dapsone D8, m/z 257.300→160.00 with DP 80V and CE 23 eV; N-Acetyl Dapsone,m/z 291.1/156.000 with DP 90V and CE 24 eV. Data processing was performed using the Analyst software (ver. 1.6.1; Applied Biosystems).

Preparation of stock and working dilution solutions

Stock solutions of Dapsone and N-Acetyl Dapsone were prepared by dissolving the drug in Acetonitrile at a concentration of 1mg/ml and stored in 5mL volumetric flasks at 2.0-8.0◦ C.Stock solution of Dapsone D8 was prepared by dissolving the drug in Acetonitrile at a concentration of 1mg/ml and stored in 10mL volumetric flasks at 2.0-8.0◦ C. Serial (working) dilutions of Dapsone was prepared at the concentrations of 125000, 100000, 50000, 25000, 12500, 2500, 100,50 and 25ng/mL using Acetonitrile:water (80:20,v/v) as diluent. Serial (working) dilutions of N-Acetyl Dapsone was prepared at the concentrations of 1000, 900, 800, 400, 200, 100, 50, 25 and 12.5ng/mL using Acetonitrile:water (80:20,v/v) as diluent. Working solution of Dapsone D8 (Internal standard) was prepared at a concentration of 1000 ng/mL using Acetonitrile:water (80:20,v/v) as diluents.

Preparation of spiked calibration and quality control samples in human plasma

Calibration curve was prepared at the concentration levels of 2500.00, 2000.00, 1000.00, 500.00, 250.00, 50.00, 2.00, 1.00 and 0.50ng/mL for Dapsone and 20.00, 18.00, 16.00, 8.00, 4.00, 2.00, 1.00, 0.50 and 0.25 for N-Acetyl Dapsone by spiking an appropriate amount of the working dilution solutions in 0.1mL blank plasma. The calibration curve was prepared and assayed along with quality control (QC) samples. QC samples were prepared in 0.1mL blank plasma at five levels of 0.50, 1.50, 250.00, 1200.00 and 2000.00ng/mL for Dapsone and 0.25, 0.75, 2.00, 10.00, and 17.50ng/mL for N-Acetyl Dapsone. The plasma samples were stored at −80 ◦ C.

Sample preparation

To an aliquot of 0.1mL plasma sample in a 5.0mL plastic centrifuge tube, add 20µL of IS working solution (1000 ng/mL Dapsone D8 solution) and vortex-mixed for 10 secs and 1mL of extraction solvent (TBME) was added and vortex-mixed for 5 min, followed by centrifugation at 4000rpm for 5min.Withdraw 0.75mL of the upper clear layer solution and evaporate at 30°C under nitrogen gas and reconstitute with 500µL of mobile phase and was transferred to an auto-sampler vial for LC–MS/MS analysis and inject 20µL of volume.

Specificity

The specificity of the method was tested by screening six different batches of blank human plasma. Each blank sample was tested for interferences in the MRM channels using the proposed extraction procedure and chromatographic/MS–MS conditions, and the results were compared with those obtained for the analytes at a concentration near to the lower limit of quantification (LLOQ).

Precision and accuracy

The intra-run precisions and accuracies were estimated by analyzing six replicates containing Dapsone and N-Acetyl Dapsone at five different QC levels, of 0.50, 1.50, 250.00, 1200.00 and 2000.00ng/m for Dapsone and 0.25, 0.75, 2.00, 10.00, and 17.50ng/mL for N-Acetyl Dapsone. The inter-run precisions were determinate by analyzing QC samples on three different runs. The criteria for acceptability of the data included accuracy within ±15% deviation (DEV) from the nominal values and a precision of within ±15% relative standard deviation.

Extraction recovery

The recovery of Dapsone and N-Acetyl Dapsone were determined by comparing the peak area obtained for QC samples that were subjected to the extraction procedure with those obtained for aqueous solution of the analytes at respective QC concentrations.

Stabilities

The stability of Dapsone and N-Acetyl Dapsone in plasma under different temperature and timing conditions was evaluated. Plasma samples were subjected to short-term conditions, to long-term storage conditions (−80◦ C), and to three freeze-thaw stability studies. The autosampler stability was conducted by re-analyzing extracted samples kept under the autosampler condition. All the stability studies were conducted using six replicates at two concentration levels (1.50 and 2000.00 ng/mL for Dapsone and 0.750 and 17.50 ng/mL for N-Acetyl Dapsone).

RESULTS AND DISCUSSION

Method Development

The electrospray ionization of Dapsone, N-Acetyl Dapsone and the IS produced the [M+H] + ions at 249.3, 291.1 and 257.3 under positive ionization conditions. In product ion spectra, ions at m/z 156.1, 156.0 and m/z 160.0 were produced as the prominent product ions for Dapsone, N-Acetyl Dapsone and the IS (Fig. 2). The quantitative analysis was performed using the MRM mode due to high selectivity and sensitive of MRM data acquisition: m/z *249.3*→156.1 for Dapsone, m/z *291.1*→156.0 for N-Acetyl Dapsone and m/z *257.3*→160.0 for the IS. Declustering potential and

collision energy were determined by observing maximum response of the product ion. The chromatographic conditions, especially the composition of the mobile phase, were optimized through several trials to achieve symmetric peak shapes for Analytes and the IS. It was found that a mobile phase containing a certain proportion of formic acid reduced the response for Analytes and the IS and a mobile phase containing high proportion of Acetonitrile gave good response for analytes and IS. It was also found that the inclusion of 2mM Ammonium acetate solution in the mobile phase was crucial to obtain high signal intensity. A flow rate of 0.8 mL/min was optimized to give a short chromatographic run time.

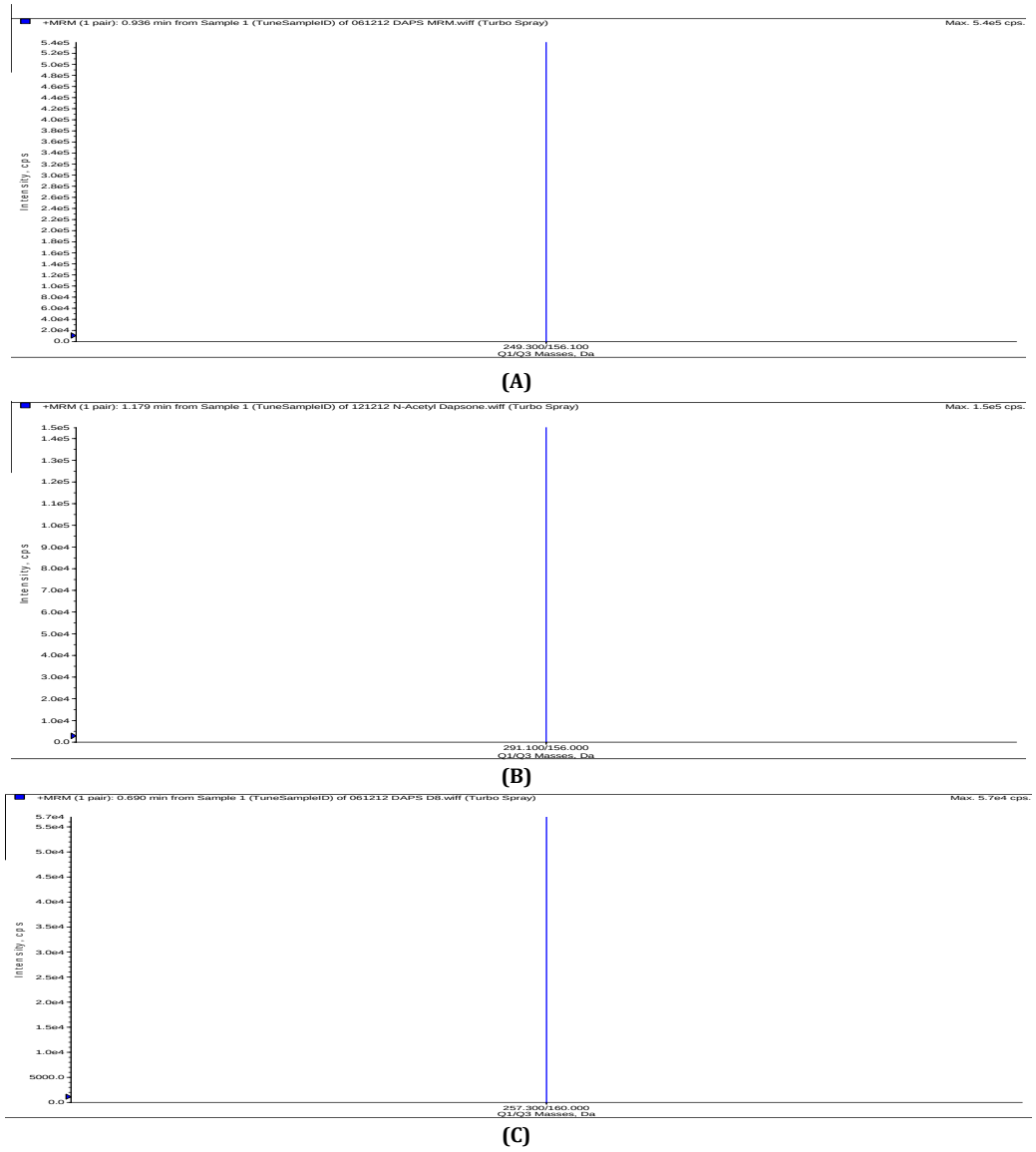

Fig. 2: MRM Scan positive mode mass spectra of (A) Dapsone, (B) N-Acetyl Dapsone and (C) Dapsone D8

Method Validation

Specificity and Selectivity

Fig. 3 showed the typical chromatograms of blank plasma, spiked plasma sample with Dapsone, N-Acetyl Dapsone and the IS. The retention time of Dapsone, N-Acetyl Dapsone and the IS was 1.80min. No significant interference in the blank plasma traces was observed from endogenous substances in drug free human plasma at the retention time of Dapsone, N-Acetyl Dapsone or the IS.

Fig no.3: (A) & (B) Blank plasma and LLOQ chromatogram of (C) Dapsone (D) N-Acetyl Dapsone and (E) Dapsone D8 (IS).

Calibration Curve

The results in Table 1 & 2. revealed a good linear correlation between nine non-zero calibration standards for Dapsone over a range of 0.50 to 2500.00 ng/mL and for N-Acetyl Dapsone over a range of 0.25 to 20.00 ng/mL. The LLOQ of Dapsone was established at 0.50 ng/mL and for N-Acetyl Dapsone it was 0.25 ng/mL, and the RSD was less than 20%. For all other calibration standards, the RSD was less than 15%.The RSD was calculated at each calibration levels between five replicates of calibration curves.

Accuracy and precision

The intra and inter-run precision and accuracy of the assay were assessed by running a single batch of samples containing a calibration curve and six replicates at each QC levels. The results, which were summarized in Table 3 & 4, demonstrated that the precision and accuracy values were within the acceptable range and the method was accurate and precise.

Extraction recovery

The extraction recovery of Dapsone and N-Acetyl Dapsone was found to be consistent and precise at all the three QC levels.The results were summarized in the Table 5 & 6.

Stabilities

Stability data were shown in Table 7 & 8. The analytes were stable in plasma under different temperature and timing conditions. Plasma samples were subjected to short-term to long-term storage conditions for 6, 21 days (−80 ◦ C), to five freeze-thaw stability, and to autosampler stability for 28 and 52 hr studies.

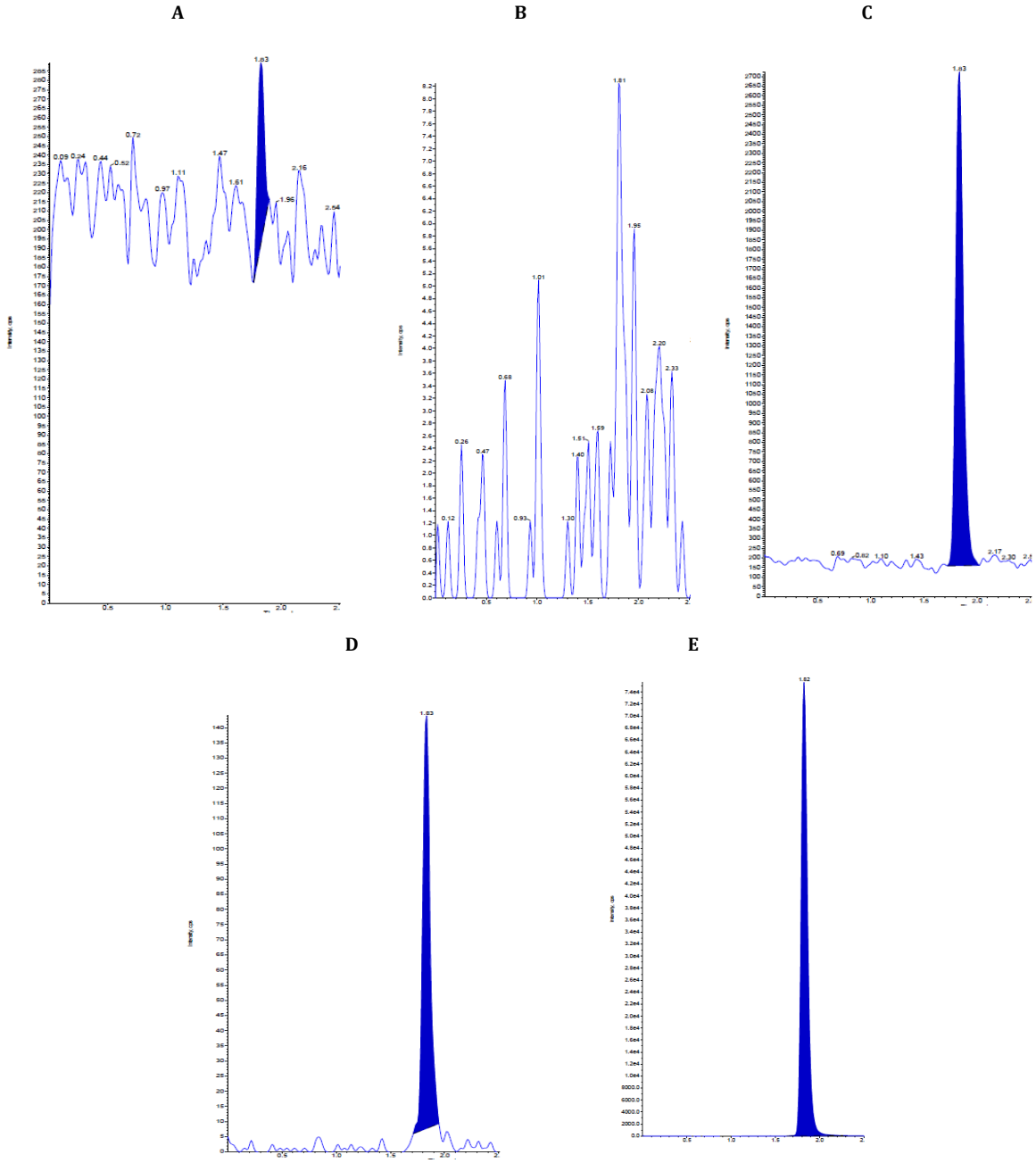

Table 1: Analysis of plasma Calibration Standards of Dapsone.

	Nominal Conc. (ng/mL)	Mean conc.[a] (ng/mL)	% CV	Accuracy %
CC1	0.5	0.4816 ± 0.01009	2.10	96.32
CC2	1	0.9864 ± 0.02420	2.45	98.64
CC3	2	2.1276 ± 0.09694	4.56	106.38
CC4	50	52.8694 ± 0.85495	1.62	105.74
CC5	250	261.8162 ± 5.65749	2.16	104.73
CC6	500	527.4496 ± 17.95610	3.40	105.49
CC7	1000	1008.5716 ± 11.79621	1.17	100.86
CC8	2000	1868.8802 ± 48.06697	2.57	93.44
CC9	2500	2371.5122 ±132.77258	5.60	94.86

[a] Data represent the mean ± S.D of five observations.

Table 2: Analysis of plasma Calibration Standards of N-Acetyl Dapsone.

	Nominal Conc. (ng/mL)	Mean conc.[a] (ng/mL)	% CV	Accuracy %
CC1	0.25	0.2646 ±0.00926	3.50	105.84
CC2	0.50	0.4792 ± 0.04938	10.30	95.84
CC3	1.00	1.0144 ± 0.04906	4.84	101.44
CC4	2.00	2.0414 ± 0.13860	6.79	102.07
CC5	4.00	4.2924 ± 0.16318	3.80	107.31
CC6	8.00	8.1080 ± 0.48461	5.98	101.35
CC7	16.00	16.6962 ± 0.29001	1.74	104.35
CC8	18.00	18.3470 ± 0.34338	1.87	101.93
CC9	20.00	21.1964 ±0.82539	3.89	105.98

[a] Data represent the mean ± S.D of five observations.

Table 3: Intra- and inter-day precision and accuracy data for Dapsone in human plasma

	Inter Day			Intra Day		
	Mean conc.[a] (ng/mL)	% CV	Accuracy %	Mean conc. (ng/mL)	% CV	Accuracy %
LLOQ QC (0.50ng/mL)	0.4788 ± 0.04280	8.94	95.76	0.5160 ± 0.02072	4.02	103.20
LQC (1.50 ng/mL)	1.4947 ± 0.06059	4.05	99.65	1.4873 ± 0.08686	5.84	99.15
MQC1(250.00 ng/mL)	246.8177 ± 7.43634	3.01	98.73	244.6103 ± 3.68720	1.51	97.84
MQC (1200.00 ng/mL)	1176.6233 ± 44.09197	3.75	98.05	1215.6612 ± 17.37612	1.43	101.31
HQC (2000.00 ng/mL)	1873.0053 ± 84.90440	4.53	93.65	1974.5425 ± 50.56393	2.56	98.73

[a] The inter- and intra-assay data represent the mean ± S.D. of 18 and 12 observations, respectively.

Table 4: Intra- and inter-day precision and accuracy data for N-Acetyl Dapsone in human plasma

	Inter Day			Intra Day		
	Mean conc.[a] (ng/mL)	% CV	Accuracy %	Mean conc. (ng/mL)	% CV	Accuracy %
LLOQ QC (0.25ng/mL)	0.2517 ± 0.02405	9.56	100.68	0.2588 ± 0.02424	9.37	103.52
LQC (0.75 ng/mL)	0.7009 ± 0.05038	7.19	93.45	0.6975 ± 0.02491	3.57	93.00
MQC1(2.00 ng/mL)	2.0195 ± 0.13812	6.84	100.98	2.0267 ± 0.12326	6.08	101.34
MQC (10.00 ng/mL)	10.4092 ± 0.81554	7.83	104.09	10.3925 ± 0.64099	6.17	103.93
HQC (17.50 ng/mL)	17.0108 ± 1.21896	7.17	97.20	16.2315 ± 0.70491	4.34	92.75

[a] The inter- and intra-assay data represent the mean ± S.D. of 18 and 12 observations, respectively.

Table 5: Extraction recovery of Dapsone from plasma

Dapsone spiked Concentration ng/mL	Dapsone concentration found (n=6)	Recovery (Mean±S.D.)%
1.50	0.83	55.24 ± 5.01
250.000	132.35	52.94 ± 9.52
1200.00	686.28	57.19 ± 9.05
2000.000	1063.40	53.17 ± 8.32

Table 6: Extraction recovery of N-Acetyl Dapsone from plasma

N-Acetyl Dapsone spiked Concentration ng/mL	N-Acetyl Dapsone concentration found (n=6)	Recovery (Mean±S.D.)%
0.75	0.37	49.82 ± 8.35
2.00	1.07	53.36 ± 9.67
10.00	4.98	49.77 ± 6.03
17.50	9.90	56.54 ± 6.52

Table 7: Stability of Dapsone under different storage conditions

Sr. No.	Storage conditions	Concentration (ng/mL)	
		Nominal	Mean found Conc. (SD)
1	Bench Top Stability for 21 Hrs	1.50	1.4123 ± 0.06752
		2000.00	1823.2510 ± 44.30751
2	Dry Extract Stability for 49 Hrs	1.50	1.4246 ± 0.00826
		2000.00	1803.1168 ± 30.46909
3	Freeze and Thaw Stability at -80.0°C (5th Cycles)	1.50	1.4355 ± 0.17512
		2000.00	1841.3651 ± 37.87052
4	Short Term Stability at -80.0°C for 6 Days	1.50	1.4413 ± 0.06746
		2000.00	1855.159 ± 43.32546
5	Auto Sampler Stability for 28 Hrs	1.50	1.4220 ± 0.01768
		2000.00	1875.5465 ±54.52139
6	Auto Sampler Stability for 52 Hrs	1.50	1.3822 ± 0.08726
		2000.00	1834.5042± 27.10693
7	Long Term Stability at -80.0°C for 21 Days	1.50	1.4447 ± 0.08232
		2000.00	1825.1615 ± 51.26578

Table 8. Stability of N-Acetyl Dapsone under different storage conditions

Sr. No.	Storage conditions	Concentration (ng/mL)	
		Nominal	Mean found Conc. (SD)
1	Bench Top Stability for 21 Hrs	0.750	0.7062 ± 0.04049
		17.50	17.1110 ± 0.83896
2	Dry Extract Stability for 49 Hrs	0.750	0.7107 ± 0.07369
		17.50	16.6463 ± 0.62794
3	Freeze and Thaw Stability at -80.0°C (5th Cycles)	0.750	0.6735 ± 0.09123
		17.50	16.5865 ± 0.84562
4	Short Term Stability at -80.0°C for 6 Days	0.750	0.6792 ± 0.04216
		17.50	16.9512 ± 0.85597
5	Auto Sampler Stability for 28 Hrs	0.750	0.7790 ± 0.04495
		17.50	17.4272 ± 0.47996
6	Auto Sampler Stability for 52 Hrs	0.750	0.7346 ± 0.02325
		17.50	17.2908 ± 0.51240
7	Long Term Stability at -80.0°C for 21 Days	0.750	0.7481 ± 0.08354
		17.50	17.0949 ± 0.31102

CONCLUSIONS

A rapid, simple and sensitive LC–MS/MS method for the simultaneous determination of Dapsone, N-Acetyl Dapsone in human plasma has been developed and validated. This method provided superior sensitivity with LLOQ as low as 0.50 ng/mL for Dapsone and 0.25 ng/mL for N-Acetyl Dapsone. The method can be successfully applied to pharmacokinetic study of Dapsone and for phenotype acetylator determination [18] using Dapsone and N-Acetyl Dapsone.

ACKNOWLEDGEMENT

The authors are indebted to Dr. Nitin Borkar, CEO of VerGo Pharma Research Ltd. and Dr. Sujal Kamble, Head of VerGo Clinicals, for their continuous support and encouragement. The authors gratefully acknowledge VerGo Clinicals Lab for providing necessary facilities to carry out this work.

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