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Sensitive and Rapid determination of Trientine and N1-Acetyl Trientine in Human Plasma by LC-MS/MS for bioequivalence study

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ABSTRACT

A simple and robust method for simultaneous determination of Trientine and N1-Acetyl Trientine in human plasma by liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed and validated. The analyte and internal standard were extracted from 200 µL plasma by liquid phase extraction. Chromatographic analysis was carried out on column Xtimate, C18 (4.6 x 50 mm) 5 µm with a flow rate of 1 mL/min, at 40˚C temperature. An isocratic elution method was applied using (A) Acetonitrile - 80% and (B) 10mM Ammonium Acetate in water - 20%. Detection and quantitation was done by multiple reactions monitoring in positive ionization with Q3 LCMS-8050, Shimadzu. Mass parameters 1035.45/1030.55 m/z and 855.15/859.50 m/z on a triple quadrupole mass spectrometer were chosen for analysis of Trientine and N1-Acetyl Trientine. Linearity was established in human plasma covering the concentration range 10.009 ng/mL to 1000.571 ng/mL for Trientine and 10.009 ng/mL to 1000.628 ng/mL for N1-Acetyl Trientine. Correlation coefficient was consistently greater than 0.99 for Trientine and N1-Acetyl Trientine using Trientine-D4 and N1-Acetyl Trientine Trihydrochloride D4 as internal standards. Different parameters such as linearity, range, precision, accuracy, ruggedness and robustness, limit of detection (LOD) and limit of quantification (LOQ) were used for a full validation of this method. The results were found to be acceptable as per the guidelines of International Conference on Harmonization (ICH), CDER, EMA[1,2,3,4,5]. The developed and validated method was successfully applied to estimate Trientine and N1-Acetyl Trientine in a bioequivalence study in healthy human volunteers. Assay reproducibility was checked by reanalysis of samples near the Cmax and the elimination phase in the pharmacokinetic profile of the drug.

Keywords: Trientine and N1-Acetyl Trientine, LC-MS/MS, Validation, ICH.

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1. INTRODUCTION

Triethylenetetramine (TETA) also known as Trientine is, a CuII-selective chelator, commonly used for the treatment of Wilson's disease, which is an autosomal recessive genetic disorder, manifested by copper accumulation in the tissues of patients. Recently, it has been shown that TETA can be used in the treatment of cancer because it possesses telomerase inhibiting and anti-angiogenesis properties. [Hea-Young Cho, Robert A. Blum, Tracey Sunderland, Garth J. S. Cooper, and William J. Jusko (2009)][6]. TETA is poorly absorbed with a bioavailability of 8 to 30%. It is widely distributed in tissues with relatively high concentrations measured in liver, heart and kidney. It is mainly metabolized via acetylation and two major acetylated metabolites exist in human serum and urine.

There are published analytical methods available in the literature for the determination of Trientine as a single analyte (Miyazaki *et al*., 1990)[7] and with metabolites in different biological samples like rat plasma, rat serum brain tissue, mouse plasma (Marc Cerrada-Gimenez *et al.* 2011)[8] and in human plasma and urine (Lu J1 *et al*., 2007)[9] and serum (Othmana *et al.,* 2007)[10]. Triethylenetetramine compound was detected and measured using High Performance Liquid Chromatography (HPLC) methods (Othmana *et al*.[10], 2007; G.J Cooper *et al*., 2008; K. Miyazaki *et al.*[7], 1990) LC-MS-MS (Lu J1 *et al*., 2007)[9] with fluorescence (Hea-Young Cho *et al*., 2009)[6] or conductometric detection (E. B. Hansen *et al*., 1985)[11]. The conductometric method has poor sensitivity, while the fluorescence derivatization methods using various labeling reagents, all generate complex chromatograms and moreover, have been optimized to detect unchanged TETA alone. The method of using 4-(1-Pyrene) butyric acid N-hydroxy succinimide ester (PSE) as a fluorescence-derivatizing agent is highly selective and sensitive but requires high temperatures for derivatization

to occur. The chromatograms obtained were complicated by the presence of many endogenous amino compounds.

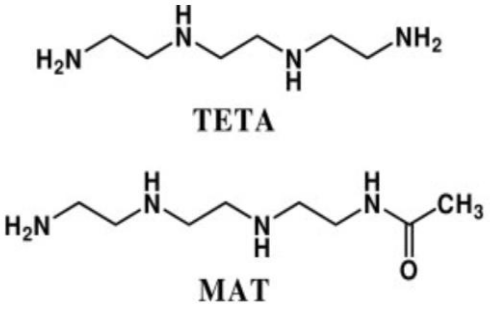


Fig: 1: Trientine (TETA) and N1-Acetyl Trientine (MAT)

The method developed by Othmana *et al.,* 2007; determined Trientine and its two metabolite in human serum using HPLC, has longer run time of 35 minutes and high limitation quantitation i.e., 0.125 mg/L for DAT and MAT and 0.0625 mg/L for TETA. The method developed by Lu J1 *et al*., 2007; determination of Trientine and its metabolite in plasma and urine using LC-MS, reported with a quantitation time of 8 min by LLE method, but has a high limit of quantitation, i.e., 0.25–16_M for TETA, MAT and DAT in plasma and urine. However, as reported the stability problem of underivatized TETA prevented the method from extending to large-scale clinical analysis for pharmacology. Serum TETA concentrations were determined using validated selective fluorometric liquid chromatographic method based on intramolecular excimer-forming derivatization and are separated within 25 minutes with limits of quantitation for serum TETA were 0.0625 mg/L (Hea-Young Cho *et al*., 2009). Refer table 1 for a comparative assessment of the reported literature.

In the present work, a sensitive, simple and rapid LC-MS/MS method was developed, optimized and validated for the simultaneous determination of Trientine and its metabolite N1-acetyltriethylenetetramine in human plasma. Advantages of this method include shorter runtime that is needed to achieve high throughput analysis required for clinical, pharmacokinetic and bioequivalence studies. In addition to lower sample processing volume and lower sample injection volume, lower limit of quantitation achieved that permit applicability of the proposed method to estimate lower concentrations of drug in human plasma. We used derivatization method and achieved the required stability. More details are included in the results section. The method was applied for a bioequivalence study of 14 healthy Indian populations. Reproducibility in the measurement of study data has been successfully demonstrated by reanalysis of incurred samples. The method was successfully applied for bioequivalence study in healthy Indian subjects with required accuracy and precision.

Table 1: Comparative assessment of chromatographic methods developed for analysis of Trientine and metabolites in human (1990-2017)

Sr. No.	Detection technique	Extraction procedure	Sample volume; injection volume	Linear range	Retention time (min); run time min	Application	Ref.
1	HPLC	LLE	10 µL human plasma	0.0625-5 mg/L (TETA) 0.125-5 mg/L (MAT and DAT)	TETA (23.7), MAT(17.7) and DAT (8.6), and HDA (16.9); 35	PK, PD, and Metabolism of Trientine in Healthy Human Participants	G.J Cooper *et al*., 2008
2	LC_MS/MS	LLE	100 µL human plasma/urine; 50 µL	0.25–16_M (TETA, MAT and DAT)	*TETA (2.3);* 8	PK measurement in one healthy subject following oral dose and 7 subjects urine samples from the diabetic patients	Lu J1 *et al*., 2007
3	HPLC	LLE with derivatization	25 µL human serum	0.0625–5 mg/L (TETA) 0.125–5 mg/L (MAT and DAT)	35	PK measurement in one healthy subject following oral dose of 600 mg.	Othmana *et al.,* 2007
4	Fluorimetric-LC	LC-based on intra molecular excimer forming derivatization	-	0.0625 mg/L - 3.0 mg/L	25	PK and PD Modeling of a Copper-Selective Chelator in Healthy Adults	Hea-Young Cho *et al*., 2009
5	HPLC-flourometric	SPE	0.5 mL plasma; 20-50 µL	40 µg/mL - 100ng/mL	-	Determination of Trientine in plasma of patients	K. Miyazaki *et al.,* 1990
6	LC-MS/MS	LLE	200 µL human plasma; 10 µL	10.009 ng/mL - 1000.571 ng/mL (TETA) 10.009 ng/mL to 1000.628 ng/mL for N1-Acetyl Trientine.	10	Bioequivalence study with 250 mg of oral administration Trientine in 14 healthy Indian subjects	PM

HPLC: High performance liquid chromatography, LC-MS/MS: Liquid chromatography- tandem mass spectrometry, LLE: Liquid-liquid extraction, SPE: Solid phase extraction, TETA: Trientine, MAT: N1-Acetyl Trientine: DAT: N1-Diacetyl Trientine , PK: Pharmacokinetic, PD: Pharmacodynamic, PM: Present method,

2. EXPERIMENTAL

2.1. Chemicals and reagents

The reference standard of Trientine (purity 99%), N1-Acetyl Trientine (purity 99.4%), Trientine-D4 (purity 99.6%), N1-Acetyl Trientine Tetramine Trihydrochloride D4 (purity 99.1%) were purchased from Vivan life sciences (P) Ltd. (Mumbai, India). Ammonium Acetate (GR/AR Grade), Methanol [HPLC Grade], Acetonitrile [HPLC Grade], Borax (AR/GR Grade), FMOC (Fluorenylmethyloxycarbonyl chloride) were purchased from Merch Specialties Pvt. Ltd. (Mumbai, India). Milli - Q / HPLC Grade Water, Human Plasma (K3EDTA), Whole Blood (K3EDTA) were utilized. Column Xtimate, C18 (4.6 x 50 mm) was purchased from Phenomenex India (Hydrebad, India). The detection was done using LCMS instrument Nexera X2 highest pressure UHPLC, [LCMS-8050, Shimadzu] and lab solutions for data processing.

2.2. Instrumentation and chromatographic conditions

The chromatography was performed on Nexera X2 UHPLC System (Shimadzu LCMS 8050) with cooling auto-sampler and column oven enabling temperature control of the analytical column. The column utilized was Xtimate, C18 (4.6 x 50 mm) 5 µm at 40˚C temperature. An isocratic elution method was applied using (A) Acetonitrile - 80% and (B) 10mM Ammonium Acetate in water - 20%. Detection was done by Q3 LCMS-8050, Shimadzu with ESI (+) ion mode for Trientine and N1-Acetyly Trientine. The auto-sampler was maintained at 5˚C and the injection volume was 10µL. Total run time for each sample analysis was 10 min. The optimized Multiple Reaction Monitoring (MRM) conditions of mass spectroscopy applied in the method development are the transitions of m/z 1035.45→1035.45 for Trientine, m/z 1039.55→1039.55 for Trientine D4, m/z 855.15→855.15 for N1-Acetyl Trientine and m/z 859.50→859.50 for N1-Acetyl Trientine D4 respectively, with a scan time of 100 ms per transition. The heat block temperature was 300˚C, nebulizing gas flow 3.0 L/min, drying gas flow 10.0 L/min, CID gas 270 kpa and HESI positive /negative interface voltage +/- 4000 volts.

Quantitation was achieved by MS/MS detection in positive ion mode using Shimdzu LCMS-8050 mass spectrometer, equipped with Heated Electro Spray Ionization (HESI) interface. Lab solution Version 6 software was used for instrument control and data acquisition.

2.3. Preparation of stock solutions calibration standards and quality control samples

A stock solution of 1.0 mg/mL concentration of Trientine, N1 Acetyl Trientine and a stock solution of 0.5 mg/mL concentration of Trientine, N1 Acetyl Trientine were prepared by dissolving requisite amount in methanol. Working solutions were prepared by diluting stock solution by methanol. Stock and working solutions were stored in refrigerator at 2 to 8°C. Calibration Standards (CSs) and Quality Controls (QCs) samples were prepared by spiking blank plasma with working solutions. The concentration of CSs were made in the range of 10-1000 ng/mL and that of QC samples were prepared at five levels (10, 28, 102, 512, 800 ng/mL) and are given in table 2.

2.4 Sample extraction procedure

To an aliquot of 200 µL of spiked plasma/subject samples, 50 µL of working solution was added and vortexed for 10 seconds. The solutions were made alkaline by adding 100 µL of 133 mM Borax solution and briefly vortexed. Added 600 µL of acetonitrile and vortexed for 3 min and then centrifuged at 4000 rpm for 10 min. The supernatant was collected, 200 µL of FMOC solution was added and vortex mixed. The samples were then kept for complete derivatization for 10 min. Following centrifugation at 4000 rpm for 5 min, the samples were collected in prelabelled autosampler vials and 10 µL was used for injection in LC-MS/MS system.

2.5. Method Validation

The method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation. The method was validated for selectivity, linearity, precision, accuracy, matrix effect, recovery and stability. [Guideline on bioanalytical method validation; EMEA/CHMP/EWP/192217/2009 Rev. 1 Corr. Committee for Medicinal Products for Human Use (CHMP) 21 July 2011, FDA Guidance; (September 2013) Guidance for Industry, Bioanalytical Method Validation].

2.5.1 Specificity and Selectivity

Specificity of the method was checked for any interferences in eight different lots of human blank plasma including one lipemic and one hemolyzed with the proposed extraction procedure. Selectivity was assessed, by comparing the chromatograms of blank plasma and plasma spiked with lowest standard from eight various sources including lipemic and hemolyzed (or donors). Cross analyte effect (cross talk) was evaluated by spiking the highest concentration individually and monitored all Multiple Reaction Monitoring (MRM) channels for interferences.

2.5.2 Precision and accuracy

All validation experiments were carried out at five (LOQQC, LQC, MQC-1, MQC-2 and HQC) QC levels. For the determining of intraday accuracy and precision, a replicate (n=6) analysis of plasma samples were performed on the same day. The inter-day accuracy & precision were assessed by analysis of three batches on different days. The precision was expressed as Relative Standard Deviation (RSD %) and accuracy as Relative Error (RE %).

2.5.3 Calibration Curve

Plotting of the peak area ratio of the transition pair of analytes to that of IS against the nominal concentration of calibration standards was done to obtain calibration curves. The concentrations used of Trientine and N1 Acetyl Trientine calibration curves were 10, 20, 50, 150, 500, 751, 900, 1000 ng/mL, while 28, 512, 800 were used for its LQC, MQC, HQC respectively. Blank sample and zero samples were run with each calibration curve. The acceptance criterion for each back-calculated standard concentration was ± 15% deviation from the nominal value except at LLOQ, which was set at ± 20%.

2.5.4 Recovery and Matrix effect

The recovery of Trientine, N1-Acetyl Trientine and ISs were determined by comparing the responses of the analytes extracted from replicate QC samples (n = 6) with the response of analytes from post extracted plasma standard sample at equivalent concentration. Recovery was determined at low, mid and high-quality control concentrations, whereas the recovery of the ISs were determined at a single concentration. The matrix of plasma constituents over the ionization of analytes and IS were determined by comparing the responses of the post-extracted plasma standard QC samples (n = 6) with the response of analytes from neat samples at equivalent concentrations. Matrix effect was determined at two levels

with six various sources of plasma (out of eight, six were normal K_3EDTA plasma, one was lipemic and one was hemolyzed with the same anticoagulant) (LQC and HQC) for Trientine and N1-Acetyl Trientine and for ISs. Dilution integrity was performed to extend the upper concentration limit with acceptable precision & accuracy.

2.5.5 Dilution Integrity

Trientine and N1-Acetyl Trientine separately spiked in human plasma samples, prepared at concentrations 2001.14 ng/mL for Trientine and 2001.256 ng/mL for N1-Acetyl Trientine, were diluted with human plasma two folds in six replicates and then analyzed.

2.5.6 Stability

Process sample stability was evaluated by re-injecting the same sample with freshly spiked calibration curve and quality control samples, which (stability samples) were stored at 5ºC for 161.48 hr. Bench top stability was evaluated for 6 hr and compared with freshly spiked plasma samples. The freeze-thaw stability was determined by comparing the stability samples that had been frozen and thawed five times, with freshly spiked quality control samples. Long term stability was evaluated by analyzing at low and high-quality control samples those were stored at -20ºC for 36 days together with freshly spiked calibration & quality control standards. All stability evaluations were based on back calculated concentrations. Samples considered to be stable if assay values were within the acceptable limits of accuracy (i.e., ±15% S.D.) and precision (i.e., 15% R.S.D.). Stability in whole blood was evaluated by spiking the known concentration at two levels in whole blood in two different aliquots. One aliquot was kept at room temperature while one was at 2-8 ºC for an hour. After an hour one more aliquot was freshly spiked in whole blood at same concentration level and all aliquots (stability) were centrifuged for plasma separation. These plasma samples were processed as per described sample preparation and their response ratio was compared.

2.6 Pharmacokinetic Application

The application of the method was demonstrated by a bioequivalence study in 14 healthy Indian subjects using a single dose of (Trientine Hydrochloride capsules USP 250 mg of an Indian Pharmaceutical Company) under fasting conditions. Each volunteer was judged to be in good health through medical history, physical examination and routine laboratory tests. The design of study comprised of a randomized, open label, single dose, two treatments, two periods, two sequence crossover bioequivalence study of Trientine Hydrochloride capsules USP 250 mg in 14 healthy human volunteers. The ethics committee approved the protocol and the volunteers provided informed written consent to participate in the study according to the principles of the Declaration of Helsinki. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA. Plasma concentration–time data of Trientine and N1-Acetyl Trientine was analyzed by non-compartmental method using Kinetica Version 4.4 (Thermo USA). Regarding AUC0–t, AUC0–∞ and Cmax, bioequivalence was assessed by means of an analysis of variance (ANOVA) and calculating the Standard 90% and Confidence Intervals (90% CIs) of the ratios test/reference (logarithmically transformed data).

Assay reproducibility was checked by reanalysis of samples near the Cmax and the elimination phase in the pharmacokinetic profile of the drug. The results were compared with initial pharmacokinetic study using the same procedure. As per the acceptance criterion at least two-third of the original results and repeat results should be within 20% of each other.

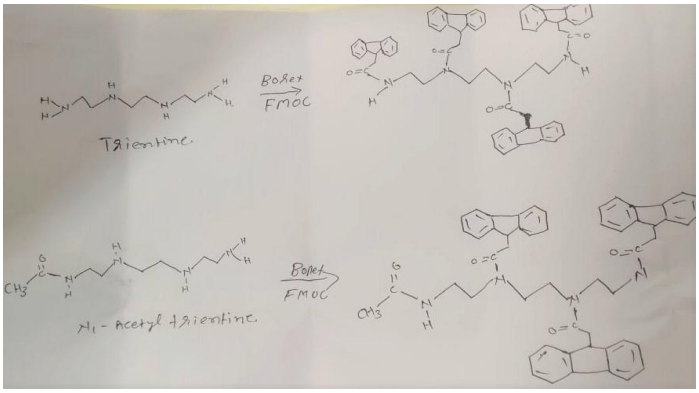
3. RESULT AND DISCUSSION

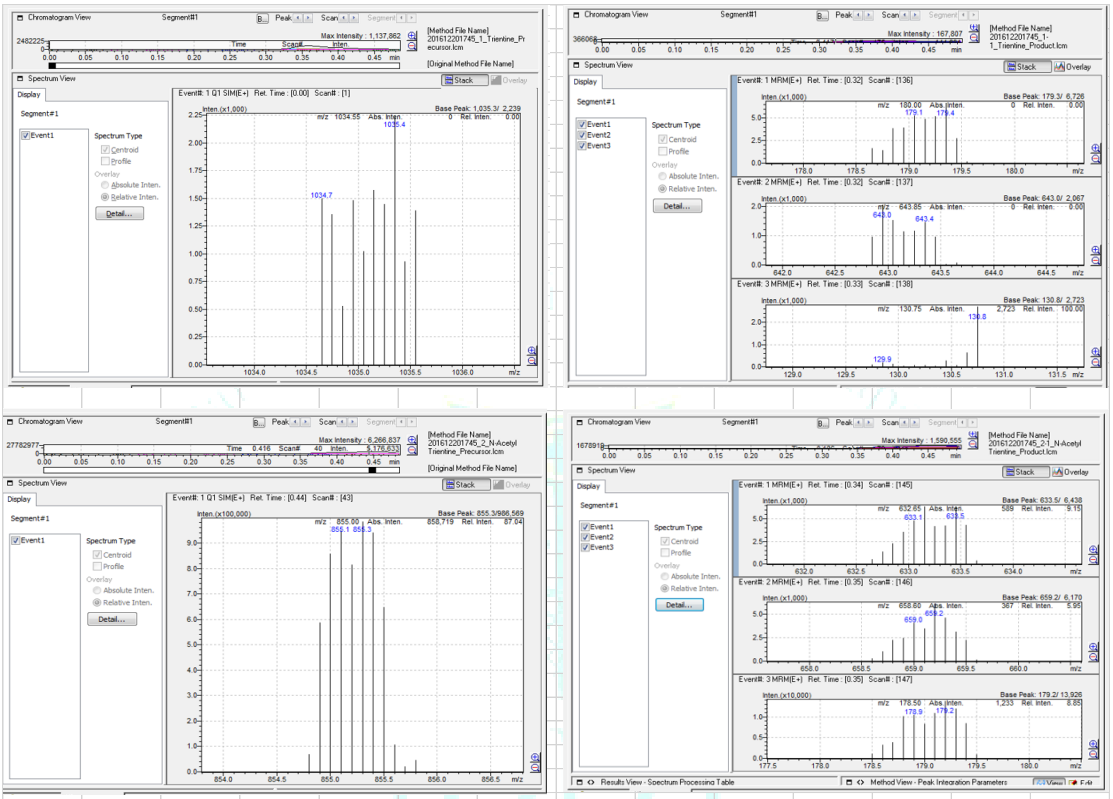
3.1 LC-MS/MS method development

There is one method reported with a less quantitation time of 8 min, however the LLOQ of the reported method (LLOQ 0.25 M) was much higher than the newly developed method. Moreover, as reported the stability problem of underivatized TETA prevented the method from extending to large-scale clinical analysis for pharmacology. The newly developed method has a much sensitive quantification of LLOQ (10.009 ng/mL) with minimum run time of 10 min. Advantages of this method include shorter runtime that is needed to achieve high throughput analysis and required for clinical, pharmacokinetic and bioequivalence studies. In addition to lower sample processing volume and lower sample injection volume, lower limit of quantitation achieved that permit applicability of the proposed method to estimate lower concentrations of the studied drug in human plasma. The method was successfully applied for bioequivalence study in healthy Indian indicant subjects with required accuracy and precision. We used derivatization method and achieved the required stability and the method was applied for a bioequivalence study of 14 healthy Indian populations. Reproducibility in the measurement of study data has been successfully demonstrated by reanalysis of incurred samples.

Flow injection method with standard solution was used to optimize ESI conditions during tuning. Full scan mass spectra was acquired for positive mode for Trientine and N1-Acetyl Trientine with their labeled internal standards respectively. Trientine and N1-Acetyl Trientine showed higher responses in positive ionization mode. The major fragments ions of these molecules were observed in the product ion scan of the collision cell. Based on the analytes properties deuterated analogs of Trientine and N1-Acetyl Trientine (Trientine D4 And N1-Acetyl Trientine trihydrochloride D4) were chosen as internal standards. Trientine and N1-Acetyl Trientine ionized efficiently in positive mode. In mass spectrometry, the parameters including temperature, flow rate of drying gas, collision gas (medium), nebulizing gas and desolvation temperature were optimized to obtain maximum response of the fragmentation. As both analyte have very low mass, i.e., 147 and 189 and its fragmentation provided unstable as well as low intensity of product mass. Which lead to high noise of baseline and reported polyamines interference the identification of the compounds because some polyamines have similar structures and molecular weights (Ref (Cooper et al, 2007). Therefore, we preferred derivatization with FMOC and the method developed in pseudo MRM mode. Parent mass was monitored by Q1 and Q3 for estimation and separated both analytes chromatographically.

Below is the structure of the analyte with derivatized analyte





Q1 and Q3 Scan results of Trientine and N1-Acetyl Trientine

The optimized Multiple Reaction Monitoring (MRM) conditions of mass spectroscopy applied in the method development are the transitions of m/z 1035.45→1035.45 for Trientine, m/z 1039.55→1039.55 for Trientine D4, m/z 855.15→855.15 for N1-Acetyl Trientine and m/z 859.50→859.50 for N1-Acetyl Trientine D4 respectively, with a scan time of 100 ms per transition. The heat block temperature was 300˚C, nebulizing gas flow 3.0 L/min, drying gas flow 10.0 L/min, CID gas 270 kpa and HESI positive /negative interface voltage +/- 4000volts.

A variety of mobile phases and columns were tried to develop a suitable method for the simultaneous estimation of Trientine and N1-Acetyl Trientine. Chromatographic conditions were optimized by using different ratios of acetonitrile-buffer and ammonium acetate in water as mobile phase using different C 18 columns i.e., chromatopak peerless basic C 18 and Xtimate C18. In case of peerless basic column, analytes signal found very less with low resolution. The best resolution with increase in signal strength for both analytes were found with welch Xtimate C18. The retention time of Trientine, Trientine D4 and N1-Acetyl Trientine, and N1-Acetyl Trientine D4 were 8.22 min, 8.22 min, 2.04 and 2.04 min, respectively. The total chromatographic run time was 10 min. The reinjection reproducibility of (%CV) in the measurement of retention time was $\leq$ 1.3%.

3.2 Assay validation results

3.2.1 Selectivity, Specificity and Linearity

The results of system suitability, autosampler and column carryover, ruggedness and dilution integrity suggest acceptable assay performance as evident from the data presented in table 2. The selectivity of the method was evident from the chromatograms of double blank plasma, plasma spiked with Trientine D4 and Trientine at different concentration level and subject sample at Cmax level. No interface due to endogenous compound was observed at the retention time of Trientine and Trientine D-4. Furthermore, none of the commonly used medications by human volunteers interfered at their retention times. The calibration curves showed good linearity over the established concentration range of 10.009 ng/mL to 1000.571 ng/mL (r^2 >0.99) for Trientine and 10.009 ng/mL to 1000.628 ng/mL for N1-Acetyl Trientine.

Representative Chromatogram of Double Blank, Blank and LLOQ Sample is provided below ;

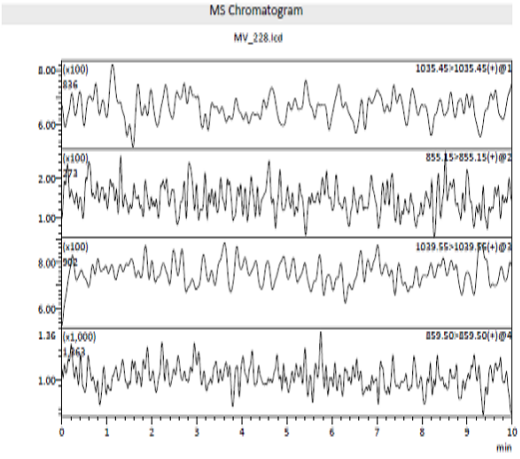


Fig 1: Double Blank

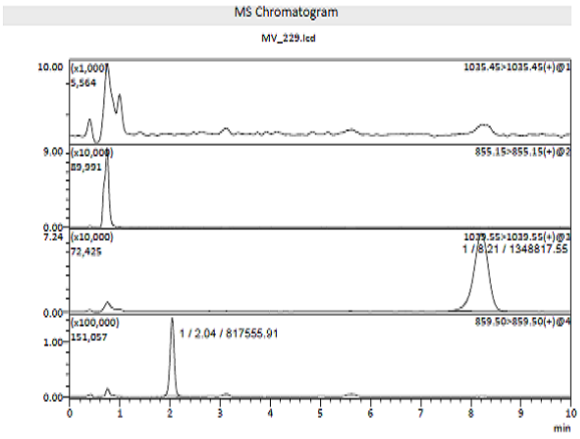


Fig 2: Blank

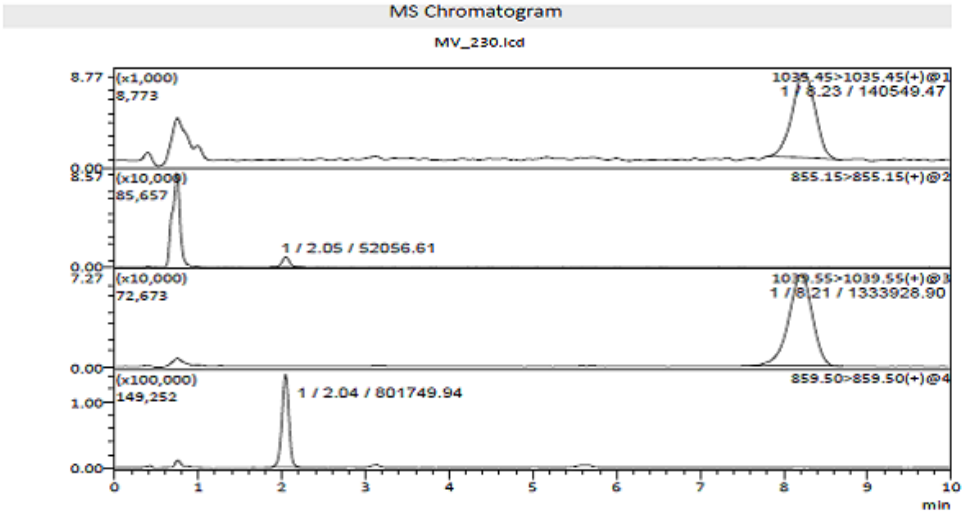


Fig 3: LLOQ

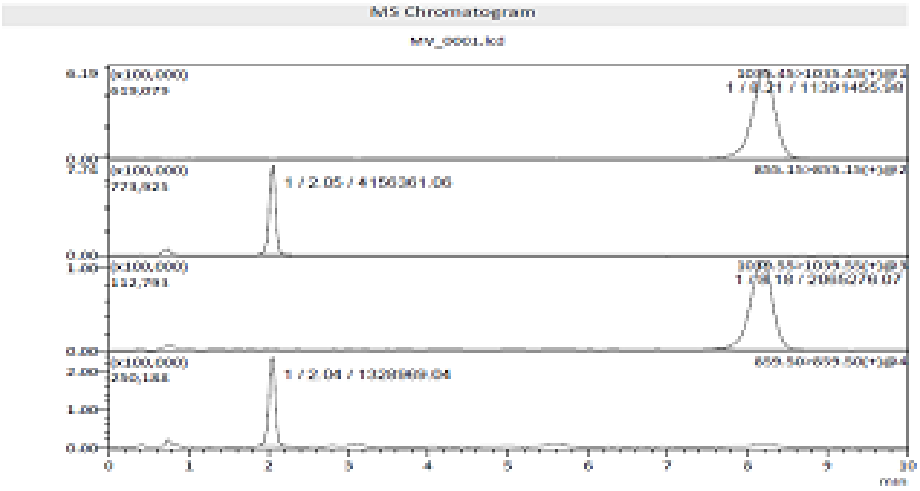


Fig. 4: Chromatographic separation of Trientine, N1-Acetyl Trientine trihydrochloride, Trientine D4 and N1-Acetyl Trientine trihydrochloride D4 (Internal Standards)

The mean value of slope, intercept, accuracy, and precision date in the measurement of calibration concentrations are shown in table 2.

Table 2: Summary of the Experimental Parameters of Trientine in K2EDTA Human Plasma

Experimental Parameters	Results	Results
Analyte	Trientine	N1-Acetyl Trientine
Biological Matrix	K2 EDTA Human Plasma	K2 EDTA Human Plasma
Specificity and Selectivity % CV	1.2%	3.5%
Analytical range	10.009 ng/mL to 1000.571 ng/mL	10.009 ng/mL to 1000.628 ng/mL
Sensitivity: Precision, Accuracy	 2.9%, 91.3%	 6.6%, 92.7%
Recovery: Trientine recovery (%) Trientine D4 recovery (%)	 93.79% 89.37%	 92.17% 89.55%
Linearity range (ng/mL)	9.745 ng/mL to 957.340 ng/mL	9.925 ng/mL to 971.109 ng/mL
Calibration Standards (ng/mL)	10, 20, 50, 150, 500, 751, 900, 1000	10, 20, 50, 150, 500, 751, 900, 1000
Quality control samples (ng/mL)	10, 28, 102, 512, 800	10, 28, 102, 512, 800
LLOQ (S/N ratio)	10.009 ng/mL (≥ 10)	10.009 ng/mL (≥ 10)
Weighing factor	1/x2	1/x2
Correlation coefficient (r^2)	≥ 0.99	≥ 0.99

3.2.2 Precision and Accuracy

Six replicates at low, medium and high-quality control concentration for Trientine and N1-Acetyl Trientine were prepared for precision and accuracy determination. The intra-day and inter-day precision (%CV) and accuracy values across the QC levels for Trientine and N1-Acetyl Trientine are tabulated in table 3 and 4 respectively. The Limit of Quantitation (LOQ) was respectively 10.009 ng/mL.

Table 3: Intra assay precision and accuracy for Trientine and N1- Acetyl Trientine.

Compound	N	Concentration (ng/mL)		%CV	Recovery (%)
		Added	Measured		
Trientine	6	10.04	11.13	2.7	110.9
	6	28.68	32.11	0.8	111.9
	6	102.45	113.5	1.2	110.8
	6	512.29	539.13	2.3	105.2
	6	800.45	817.97	1.4	102.2
N1 Acetyl Trientine	6	10.03	11.09	3.8	110.5
	6	28.67	32.90	3.0	114.7
	6	102.41	115.46	4.2	112.7
	6	512.06	569.26	3.7	111.2
	6	800.09	861.27	4.3	107.6

Table 4: Inter-assay precision and accuracy for Trientine and N1- Acetyl Trientine.

Compound	N	Concentration (ng/mL)		%CV	Recovery (%)
		Added	Measured		
Trientine	18	10.04	10.32	6.7	102.8
	18	28.68	30.93	3.0	107.8
	18	102.45	111.05	1.8	108.4
	18	512.29	527.37	2.4	102.9
	18	800.54	804.10	2.0	100.5
N1 Acetyl Trientine	18	10.03	10.51	4.9	104.8
		28.67	31.28	4.4	109.1
		102.41	112.02	3.6	109.4
		512.06	540.28	4.6	105.5
		800.09	838.80	3.5	104.8

3.2.3 Extraction Recovery

The % recovery and extraction recovery for Trientine and N1-Acetyl Trientine are presented in table 5. Six replicates at low, medium and high, quality control concentration for Trientine and N1-Acetyl Trientine were prepared for recovery determination. The mean recovery was 88.11%, 94.38% and 98.87% at HQC, MQC & LQC level respectively for Trientine and 88.22%, 92.50% and 95.79% at HQC, MQC & LQC level respectively for N1-Acetyl Trientine. The mean recovery was 89.37% and 89.55% for IS Trientine D4 and N1-Acetyl Trientine D4 respectively. The presence of unmonitored, co-eluting compounds from the matrix can be directly impact the overall performance of a validated method. It is necessary to evaluate MFs to assess the matrix effect. Matrix effect was also checked in lipemic and hemolyzed plasma samples together with normal K_3EDTA plasma. This was determined by examining the precision (%CV) values of the slopes of the calibration curves prepared from eight different plasma lots, which included six K_3EDTA, one lipemic and one hemolyzed plasma samples. Average matrix factor values (matrix factor = response of post-spiked concentrations/ response of neat concentrations) obtained for Trientine and N1-Acetyl Trientine were 0.96 (CV 2.1%, n = 6+2) and 0.94 (CV 2.1%, n = 6+2) at LQC and 1.0 (CV 2.1%, n = 6+2) and 1.0 (CV 3.0%, n = 6+2) for HQC, respectively,

whereas on ISs it was found to be 0.96 (CV 5.1%, n = 6+2) for Trientine D4 & 1.0 (CV 5.0%, n = 6+2) for N1-Acetyl Trientine D4 at tested concentration of 500 ng/mL & 100 ng/mL respectively. There were no significant matrix effects observed for any of the analytes or the ISs.

Table 5: Recovery (%) and Extraction recovery for Trientine and N1- Acetyl Trientine for human plasma.

Recover (%)	Trientine (%)	Precision (%)	N1- Acetyl Trientine (%)	Precision (%)	Trienetine D4 (%)	N1- Acetyl Trientine D4 (%)
High	98.60	1.9	98.52	3.3	101.59 (precision 1.8)	101.46 (precision 3.3)
Medium	105.04	2.4	103.17	3.4		
Low QC	100.86	2.6	100.0	5.7		
Mean recovery	101.50	-	100.56	-	101.59	101.46
Extraction Efficiency (Area) (%)						
High	88.11	0.8	88.22	4.1	89.37 (precision 2)	89.55 (precision 3)
Medium	94.38	7.8	92.50	8.8		
Low QC	98.87	3.3	95.79	6.7		
Mean recovery	93.79	-	92.17	-	89.37	89.55

3.2.4 Dilution Integrity (DI)

Dilution integrity for Trientine was evaluated by preparing DI samples with about 2 times the concentration of the high CC. Take 0.25 mL volume from drug dilution (200114.15 ng/mL for Trientine and 2000125.59 ng/mL for N1 Acetyl Trientine) and spike of this solution in plasma to get final concentration of 2001.14 ng/mL. This was diluted to 1/5th and 1/10th, processed as per standard test method and analyzed against a calibration curve.

The accuracy of both the dilutions (1/5th and 1/10th) were 102.7% and 105.2% for Trientine and 98.0% and 101.8% for N1 Acetyl Trientine, which is within the acceptance range of %CV <15%. The precision of the samples for both the dilutions (1/5th and 1/10th) were 1.7% and 1.4% for Trientine and 8.5% and 6.7% for N1-Acetyl Trientine, which is within the acceptance range of %CV <15%.

3.2.5 Sample Stability

Stock solutions kept for short term and long-term stability as well as spiked plasma solutions showed no evidence of degradation under all the studied conditions. The stability of the analytes in human plasma under different temperature and timing conditions were evaluated. QC samples were subjected to long-term storage conditions (-20°C) and to freeze–thaw stability studies. All the stability studies were conducted at two concentration levels with six determinations for each. For process stability, the results indicated that the difference in the back-calculated concentration are stable at least for 161:48 hr at 5°C in the auto-sampler. The % mean ratio for the Low and High QCs were 90.01% and 91.98% for Trientine and 90.39% and 93.93% for N1-Acetyl Trientine, which is within acceptable range of 85%-115%.

For bench top stability, the results allowed us to conclude that both analytes are stable for at least 6:45 hr at room temperature in plasma samples. The % mean ratio for the Low and High QCs were 100.26% and 98.22% for Trientine and 98.07% and 96.86% for N1-Acetyl Trientine. Freeze and thaw stability results indicated that the repeated freeze and thawing (five cycles) did not affect the stability of Trientine And N1-Acetyl Trientine. Long-term stability of the analytes in plasma at -20°C was found to be stable for at least 36 days. The % mean ratio for the high and low QCs were 105.10% and 103.87% for Trientine and 102.83% and 97.78% for N1-Acetyl Trientine, which is within acceptable range of 85%-115%. For blood sample stability the % change for stability samples was < 5% from the comparison samples for Trientine And N1-Acetyl Trientine at LQC & HQC level.

3.3 Application to a Bioequivalence Study

Blood samples were obtained following oral administration of Trientine Hydrochloride capsules USP 250 mg into K_3EDTA vacutainer solution as an anticoagulant. Plasma was separated by centrifuging the blood using an eppendorf centrifuge at 4000 rpm for 5 min and stored frozen at −20±5°C until analysis. An aliquot of 0.2 mL of thawed plasma samples were spiked with IS and processed. Plasma concentration–time data of Trientine and N1-Acetyl Trientine was analyzed by non-compartmental method using Kinetica Version 4.4 (Thermo USA). Regarding AUC0–t, AUC0–∞ and Cmax, bioequivalence was assessed by means of an Analysis of Variance (ANOVA) and calculating the standard 90% Confidence Intervals (90% CIs) of the ratios test/reference (logarithmically transformed data). The mean plasma concentration-time curves for the two formulations are represented in Fig. 5. Pharmacokinetic parameters derived from these curves are presented in table 6.

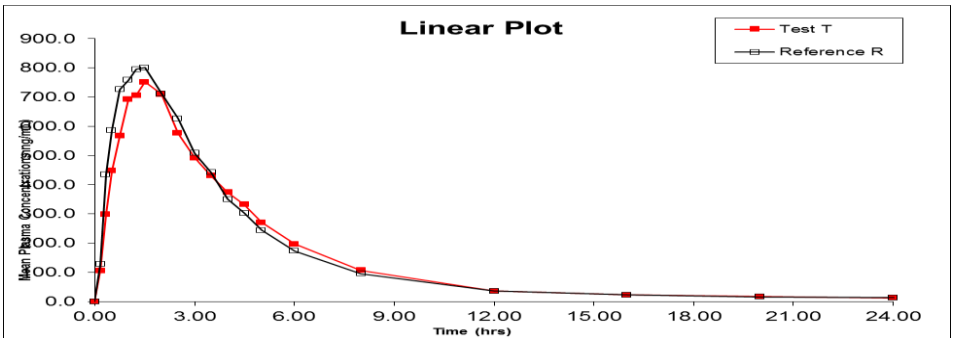


Fig 5: Mean plasma concentration-time linear plots of Trientine after administration of (test and reference) Trientine formulation to 14 healthy Indian Subjects

Table 6: Pharmacokinetic parameters of Trientine and N1-Acetl Trientine following oral administration of one Trientine Hydrochloride capsules USP 250 mg

Pharmacokinetic Parameters	Geometric Mean (%CV) Arithmetic Mean ± SD			
	Trientine		N1-Acetyl Trientine	
	Test Formulation (n=14)	Reference Formulation (n=14)	Test Formulation (n=14)	Reference Formulation (n=14)
Cmax (ng/mL)	798.60 (41.11) 901.11 ± 267.43	721.32 (53.46) 824.00 ± 463.17	727.36 (33.21) 764.08 ± 267.53	707.17 (26.99) 796.38 ± 228.63
AUC(0-t) (ng.h/mL)	2865.10 (42.82) 3210.22 ±1399.19	2533.10 (44.63) 3014.12 ± 1875.38	6823.02 (22.17) 7101.36 ± 2263.26	6654.54 (23.76) 6746.21 ± 2359.39
AUC(0-inf) (ng.h/mL)	3138.11 (44.75) 3346.40 ±1776.39	2903.31 (64.34) 3103.77 ± 2237.43	7364.11 (29.03) 7893.33 ± 3318.67	7012.31 (35.84) 7503.32 ± 3908.62
% AUCextra	4.12 (53.43) 5.43 ± 3.67	3.367 (79.01) 3.90 ± 4.36	5.21 (40.12) 5.88 ± 3.47	5.06 (33.92) 5.22 ±3.87
Tmax (h)*	1.38 (0.17 -3.00)	1.26 (0.50- 2.00)	5.00 (3.50 -6.00)	4.75 (3.00- 6.00)

4 CONCLUSIONS

The described LC-MS/MS method provides fast, sensitive and selective procedure for simultaneous determination of Trientine And N1-Acetyl Trientine in human plasma. The analytes in this method were free from ion suppression effects generated by matrix. As an interference free and sensitive method to be used for bioequivalent study applicability we have adopted a psudo method. From the results of all the validation parameters, we can conclude that the present method can be useful for bioequivalence studies with desired precision and accuracy. The method can be useful for the analysis of larger number of samples as it uses a sample extraction procedure and requires low sample volume is highly selective and has a short assay time.

Conflicts of interest: The author declares no conflict of interests.

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Appendix A. Supporting information: Supplementary data associated with this article can be found in the online version

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