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ESTIMATION OF TOTAL IRON AND TRANSFERRIN BOUND IRON (TBI) IN HUMAN SERUM USING ICP-OES AND ITS APPLICATION ON BIOEQUIVALENCE STUDIES OF IRON-SUCROSE INJECTION

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INTRODUCTION

1.6 Billion people are affected by Iron-deficiency anemia worldwide. IV Iron therapy commonly administered to dialysis patients as an adjunct to managing anemia provides a potentially rich source for intradialytic bioactive Iron. Iron-Sucrose, Iron-dextran or ferric gluconide complex like venofer or Inferon is used to treat Iron deficiency primarily in patients on chronic dialysis and also in patients unable to take oral iron supplements. After parenteral administration, the concentration of total Iron remain substantially increased. Most colorimetric analysis of Iron detect only small quantities of Iron-sucrose in serum because Iron-sucrose is stable in acidic media & Iron chelating cannot dissociate iron from sucrose or dextran. Development of these pharmaceutical agents requires pharmacokinetic studies monitoring levels of both administered agent & transferrin bound Iron. Accurate quantitative detection of serum Iron components in the presence of Iron carbohydrates have proven problematic. Iron Sucrose present in serum tends to interfere with conventional techniques for TBI detection that utilize colorimetric or Spectrophotometry. Such techniques require reduction of ferric ion to ferrous ion & Chelation of ferrous iron to form a colored complex. These procedures fail to accurately distinguish between Iron originating from Iron carbohydrate and Iron originating from TBI. Conventional detection techniques requiring ferric reduction not only release Iron from TBI but also release Iron from Iron carbohydrate complex. This results in overestimation of TBI present in serum. An inaccurate measurement of TBI may cause false detection of TBI over saturation indicating existence of free Iron, a condition that may cause acute toxicity.

A need therefore exists for a method & equipment for isolation of Iron components in serum that contain Iron-Sucrose complex. Further, We also need a method for isolating and directly measuring amount of TBI and the amount of Iron carbohydrate present in the serum after administration of Iron-sucrose complex. Pharmacokinetic studies of IV Iron sucrose are complicated by background circulating iron levels as well as need to differentiate and independently monitor Iron-sucrose and TBI. Prior to administration of product, most circulating Iron is in the form of TBI. Following IV therapy, circulating Iron is both as component of dosed formulation & complexed to transferrin. The typical colorimetric assays are incapable of discriminating and accurately quantifying TBI or total iron levels following iron sucrose administration. The measurement of TBI requires separating & excluding free Iron and drug derived iron from TBI.

We have developed and validated a robust and accurate method for analysis of total Iron and TBI to generate Pharmacokinetic data as component of Bioequivalence studies for generic IV. The measurement device used for total Iron and Transferrin Bound Iron was ICP-OES iron from Iron sucrose complex..Circulating Iron-Sucrose could then be calculated as difference between total

MATERIALS

Iron solution, 1G/dl used as Standard was purchased from Fisher scientific while Indium Standard, 1G/dl purchased from Fluka.

Sodium Nitrate, Sodium Chloride purchased from Merck, Cysteine Hydrochloride Monohydrate purchased from Lobachemicals and Calcium Nitrate Tetrahydrate purchased from SDFCL were used for preparation of Simulated Serum.

Holo transferrin Human purchased from sigma was used for preparation of TBI QCs. Nitric acid purchased from Merck while HPLC water purchased from Rankem.

Alumina Basic Cartridges, 500mg/3 mL Extraction cartridges purchased from Orochem.

EQUIPMENT

A Thermo Scientific Inductive Couple Plasma System Equipped with Optical Emission Spectrophotometer (7000 Series) and System controlled through Qtegra Software.

ICP-OES Condition:

View Direction	Axial
UV Exposure Time	15
UV RF Power	1150
UV Neb Gas Flow	0.5
VIS Exposure Time	5
VIS RF Power	1150
VIS Nebulizer Gas Flow	0.5
Cool Gas Flow rate	12
Auxiliary Gas Flow Rate	0.5
Iron Wavelength	259.940
Indium wavelength	325.609

METHOD VALIDATION

Method Used for Determination of Iron and Transferrin Bound Iron in Human plasma was developed and Validated for ICP-OES.

TOTAL IRON

- Method Validated in terms of System Suitability, Specificity, Matrix effect, Linearity, Accuracy & Precision, Extraction Efficiency, Dilution Integrity and Stability experiment.
- System suitability performed by aspirating 6 replicate of higher standard.
- Calibration standard used for linearity were prepared in Simulated serum.
- QCs were used for Accuracy & Precision determination were prepared in Human serum and Simulated serum.
- Specificity was evaluated by using 4 normal serum as well as 1 Hemolytic and Lipimic Serum.
- Matrix Effect was evaluated at HQC & LQC level using 4 normal serum as well as 1 Hemolytic and Lipimic Serum.
- Extraction efficiency was evaluated by comparison of Concentration of HQC, MQC and LQC level in Simulated serum as compare with concentration of HQC, MQC and LQC in Human Serum.
- Dilution Integrity evaluated for dilution Factor 2 and 5 times of High QC.
- Stock Solution Stability Performed at HQC and LQC level at –20±5 °C.
- Freeze thaw stability performed at HQC and LQC level in Human serum and Simulated Serum at –20±5 °C.
- Bench Top stability performed at HQC and LQC Level in Human Serum and Simulated Serum at RT.
- Long Term stability performed at HQC and LQC level in Human Serum at –20±5 °C

TBI

- Method Validated in terms of System Suitability, Linearity, Accuracy & Precision, Extraction Efficiency, Dilution Integrity and Stability experiment.
- Holo Transferrin Human was added into Human Serum.
- System suitability performed by aspirating 6 replicate of higher standard.
- Calibration standard used for linearity were prepared in Simulated serum.
- QCs were used for Accuracy & Precision determination were prepared in Human serum.
- Extraction efficiency was evaluated by comparison of Concentration of HQC, MQC and LQC level in Simulated serum as compare with concentration of HQC, MQC and LQC in Human Serum.
- Dilution Integrity evaluated for dilution Factor 2 and 5 times of High QC.
- Stock Solution Stability Performed at HQC and LQC level at –20±5 °C.
- Freeze thaw stability performed at HQC and LQC level in Human serum at –20±5 °C.
- Bench Top stability performed at HQC and LQC Level in Human Serum at RT.

RESULTS

TOTAL IRON

SPECIFICITY

No significant interference is observed from concomitant analyte at the wave length of Iron.

MATRIX EFFECT

% CV for Mean of Matrix Factor for HQC-1.1% & LQC-0.4 %

% CV of ISTD Normalized Factor for HQC-1.1% & LQC-1.5 %

LINEARITY

Method was found Linear from 25.250 µg/dL to 2010.000 µg/dL in Simulated Serum

Correlation coeffiecnt > 0.98

% Accuracy for CC Standard range: 98.2 % to 105.1 %

% CV for CC Standard range: 0.6 % to 3.6 %

Standard ID	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	r^2
Nominal (µg/dL)	25.250	101.000	202.000	404.000	808.000	1206.000	1608.000	2010.000	
P&A I	26.700	97.600	197.900	405.400	816.600	1179.600	1609.100	2021.800	0.9999
P&A III	27.400	102.600	200.000	398.300	808.800	1169.500	1669.900	1983.300	0.9992
P&A V	25.500	101.900	203.200	407.200	818.500	1203.100	1611.000	2004.300	1.0000
Mean	26.533	100.700	200.367	403.633	814.633	1184.067	1630.000	2003.133	
±SD	0.961	2.707	2.669	4.706	5.140	17.240	34.567	19.276	
%CV	3.6	2.7	1.3	1.2	0.6	1.5	2.1	1.0	
%Accuracy	105.1	99.7	99.2	99.9	100.8	98.2	101.4	99.7	

ACCURACY & PRECISISON

HUMAN SERUM

Intra Day Accuracy: 95.0 % to 108.1 % (H, M, L)
Intra Day Accuracy: 98.2 % to 116.3 % (LL)
Intra Day Precision: 0.29 % to 0.91 % (H, M, L)
Intra Day Precision: 1.32 % to 2.90 % (LL)
Inter Day Accuracy: 99.5 % to 102.6 % (H, M, L)
Inter Day Accuracy: 108.9 % (LL)
Inter Day Precision: 3.5 % to 4.4 % (H, M, L)
Inter Day Precision: 7.6 % (LL)

SIMULATED SERUM

Intra Day Accuracy: 93.7 % to 104.6 % (H, M, L)
Intra Day Accuracy: 103.2 % to 118.4 % (LL)
Intra Day Precision: 0.43 % to 1.09 % (H, M, L)
Intra Day Precision: 1.21 % to 5.14 % (LL)
Inter Day Accuracy: 98.7 % to 99.5 % (H, M, L)
Inter Day Accuracy: 109. 6 % (LL)
Inter Day Precision: 3.2 % to 4.5 % (H, M, L)
Inter Day Precision: 6.9 % (LL)

Intra-day & Inter-day Precision & Accuracy for Total Iron in Simulated Serum

	H QC			M QC			L QC			LLOQ QC		
	Nominal concentration(µg/dL)											
	1608.000			964.800			252.390			25.240		
Sr. No.	P&A I	P&A III	P&A V	P&A I	P&A III	P&A V	P&A I	P&A III	P&A V	P&A I	P&A III	P&A V
1	1604.300	1525.000	1640.600	958.400	928.900	1003.600	252.800	239.400	263.200	28.700	26.100	26.000
2	1587.500	1531.600	1636.600	964.800	918.300	1013.100	253.000	238.800	263.600	29.800	26.800	26.300
3	1592.600	1545.700	1672.600	948.700	917.300	1013.700	251.600	236.600	261.900	32.900	27.100	26.100
4	1597.500	1522.600	1635.500	961.200	911.800	1014.900	253.000	235.500	263.700	28.900	27.400	26.400
5	1585.600	1529.600	1636.400	945.500	914.000	1014.000	256.300	234.200	263.000	29.600	27.200	26.000
6	1592.500	1495.200	1629.200	952.900	908.700	997.400	251.500	234.700	260.100	29.400	27.700	25.500
Mean	1593.333	1524.950	1641.817	955.250	916.500	1009.450	253.033	236.533	262.583	29.883	27.050	26.050
±SD	6.822	16.655	15.522	7.484	7.026	7.228	1.740	2.156	1.376	1.535	0.554	0.315
%CV	0.43	1.09	0.95	0.78	0.77	0.72	0.69	0.91	0.52	5.14	2.05	1.21
% Accuracy	99.1	94.8	102.1	99.0	95.0	104.6	100.3	93.7	104.0	118.4	107.2	103.2
Inter-day (Global Data)												
Mean	1586.700			960.400			250.717			27.661		
±SD	50.987			39.813			11.199			1.898		
%CV	3.2			4.1			4.5			6.9		
% Accuracy	98.7			99.5			99.3			109.6		

Intra-day & Inter-day Precision & Accuracy for Total Iron in Human Serum

	H QC			M QC			L QC			LLOQ QC		
	Nominal concentration(µg/dL)											
	1608.000			964.800			252.4			25.3		
Sr. No.	P&A I	P&A III	P&A V	P&A I	P&A III	P&A V	P&A I	P&A III	P&A V	P&A I	P&A III	P&A V
1	1628.100	1535.100	1670.500	1051.700	959.000	980.600	273.800	247.900	253.200	28.000	28.100	25.300
2	1623.500	1526.900	1644.900	1029.700	955.800	980.900	271.800	248.800	254.900	29.100	28.000	24.700
3	1633.300	1545.700	1658.400	1047.000	949.000	989.000	273.400	246.700	257.200	30.500	28.800	24.900
4	1619.700	1519.700	1638.300	1042.500	942.900	986.400	273.000	247.000	255.500	29.500	28.200	25.300
5	1605.600	1521.200	1662.300	1029.600	944.500	977.600	273.400	243.500	259.300	29.400	28.400	25.100
6	1616.500	1518.400	1642.700	1039.000	937.900	975.500	272.100	246.200	253.500	30.000	28.900	23.800
Mean	1621.117	1527.833	1652.850	1039.917	948.183	981.667	272.917	246.683	255.600	29.417	28.400	24.850
±SD	9.664	10.696	12.724	9.026	8.036	5.147	0.796	1.813	2.320	0.852	0.374	0.565
%CV	0.60	0.70	0.77	0.87	0.85	0.52	0.29	0.73	0.91	2.90	1.32	2.27
%Accuracy	100.8	95.0	102.8	107.8	98.3	101.7	108.1	97.7	101.3	116.3	112.3	98.2
Inter-day (Global Data)												
Mean	1600.600			989.922			258.400			27.556		
±SD	55.585			39.646			11.328			2.099		
%CV	3.5			4.0			4.4			7.6		
%Accuracy	99.5			102.6			102.4			108.9		

Dilution Integrity

% Accuracy: 100.4 % for D/2 and 100.3 % for D/5

% CV: 0.5 % for D/2 and 0.3 % for D/5

Dilution Integrity for total iron in Human Serum

Sr. No.	1/2nd Dilution — Nominal Concentration (µg/dl.) 1608.000 — Dilution Factor 02	
	Diluted Concentration	Actual Concentration
1	806.900	1613.800
2	812.600	1625.200
3	805.000	1610.000
4	803.200	1606.400
Mean		1613.850
± SD		8.148
% CV		0.5
%Nominal		100.4

Dilution Integrity for total iron in Human Serum

Sr. No.	1/5th Dilution — Nominal Concentration (µg/dl.) 1608.000 — Dilution Factor 05	
	Diluted Concentration	Actual Concentration
1	321.100	1605.500
2	323.100	1615.500
3	323.200	1616.000
4	323.200	1616.000
Mean		1613.250
± SD		5.172
% CV		0.3
%Nominal		100.3

TBI

LINEARITY

Method was found Linear from 9.100 µg/dL to 2010.000 µg/dL in Simulated Serum

Correlation coeffiecnt > 0.98

% Accuracy for CC Standard range: 88.6 % to 106.2 %

% CV for CC Standard range: 1.4 % to 13.5 %

Standard ID	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	r^2
Upper limit	10.920	26.105	65.205	130.410	260.820	521.640	869.400	1242.000	
P&A VI	9.800	23.000	56.200	113.800	225.600	448.100	745.200	1090.100	0.9999
P&A VIII	8.500	-	49.300	94.800	183.800	380.600	734.000	1137.300	0.9963
P&A IX	10.700	19.900	64.600	94.400	193.700	440.500	754.100	1095.400	0.9993
Mean	9.667	21.450	56.700	101.000	201.033	423.067	744.433	1107.600	
±SD	1.106	2.192	7.662	11.087	21.844	36.973	10.072	25.857	
%CV	11.4	10.2	13.5	11.0	10.9	8.7	1.4	2.3	
%Accuracy	106.2	94.5	100.0	89.1	88.6	93.3	98.5	102.6	

ACCURACY & PRECISISON

Intra Day Accuracy: 95.1 % to 102.5 % (H, M, L)
Intra Day Precision: 0.75 % to 15.74 % (H, M, L)
Inter Day Accuracy: 99.5 % to 101.2 % (H, M, L)
Inter Day Precision: 6.9 % to 11.2 % (H, M, L)

Intra-day & Inter-day Precision & Accuracy for TBI in Human Serum

	H QC			M QC			L QC		
	Nominal concentration(µg/dl.)								
	864.000			558.100			25.600		
Sr. No.	P&A VI	P&A VIII	P&A IX	P&A VI	P&A VIII	P&A IX	P&A VI	P&A VIII	P&A IX
1	897.000	696.100	837.400	565.800	449.900	563.600	19.400	21.100	17.100
2	889.700	1013.000	822.900	558.700	710.700	561.100	24.800	24.300	26.800
3	879.300	867.200	837.700	562.800	566.600	559.800	25.400	25.100	26.500
4	877.900	841.600	836.500	553.500	564.700	560.200	25.600	26.000	26.800
5	881.600	852.200	818.300	558.900	564.700	556.300	25.700	26.200	26.800
6	881.600	870.200	869.300	558.200	574.700	578.000	25.100	26.300	27.100
Mean	884.517	856.717	837.017	559.650	571.883	563.167	24.333	24.833	25.183
±SD	7.358	100.760	17.848	4.222	82.812	7.639	2.439	1.984	3.965
%CV	0.83	11.76	2.13	0.75	14.48	1.36	10.02	7.99	15.74
%Accuracy	102.4	99.2	96.9	100.3	102.5	100.9	95.1	97.0	98.4
Inter-day (Global Data)									
Mean	859.417			564.900			24.783		
±SD	59.141			45.469			2.768		
%CV	6.9			8.0			11.2		
%Accuracy	99.5			101.2			96.8		

DILUTION INTEGRITY

% Accuracy: 97.0 % for D/2 and 92.0% for D/5

% CV: 0.9 % for D/2 and 0.5 % for D/5

Dilution Integrity For TBI in Human Serum

Sr. No.	Nominal Concentration (µg/dl.) 864.000 — Dilution Factor 2	
	Diluted Concentration	Actual Concentration
1	416.300	832.600
2	418.800	837.600
3	424.500	849.000
4	416.400	832.800
Mean		838.000
± SD		7.689
% CV		0.9
%Nominal		97.0

Dilution Integrity For TBI in Human Serum

Sr. No.	Nominal Concentration (µg/dl.) 864.000 — Dilution Factor 5	
	Diluted Concentration	Actual Concentration
1	158.600	793.000
2	159.800	799.000
3	159.300	796.500
4	158.100	790.500
Mean		794.750
± SD		3.753
% CV		0.5
%Nominal		92.0

EXTRACTION EFFICIENCY

HQC		MQC		LQC		ISTD	
% Recovery	% CV	% Recovery	% CV	% Recovery	% CV	% Recovery	% CV
94.728	3.0	102.410	2.1	103.608	7.3	83.873	0.8

STOCK SOLUTION STABILITY

Stock solution stable for 3 days at 20±5 ° C

Mean Ratio in LQC-105.320 % and HQC-93.961 %

BT STABILITY

Bench Top stability of HQC and LQC accepted for 3.30 Hrs at RT

Mean Ratio in HQC-100.207 % and LQC-103.645 %

FT STABILITY

Freeze Thaw stability of HQC and LQC accepted upto 3 cycles

Mean Ratio in HQC-104.778 % and LQC-98.036 %

RESULTS

TOTAL IRON

EXTRACTION EFFICIENCY

HQC		MQC		LQC		ISTD	
% Recovery	% CV	% Recovery	% CV	% Recovery	% CV	% Recovery	% CV
100.195	0.8	103.457	0.3	104.294	0.5	96.409	1.1

STOCK SOLUTION STABILITY

Stock solution stable for 12 days at 20±5 ° C

Mean Ratio in HQC-101.653 % and LQC-93.480 %

BT STABILITY

Bench Top stability of HQC and LQC accepted for 6.51 Hrs for Simulated Serum and 7.38 Hrs for Human Serum at RT

Mean Ratio in HQC-99.746 % and LQC-100.673 % -Simulated Serum

Mean Ratio in HQC-98.209 % and LQC-94.295 % -Human Serum

FT STABILITY

Freeze Thaw stability of HQC and LQC accepted upto 3 cycles

Mean Ratio in HQC-95.292 % and LQC-98.824 % -Human Serum

Mean Ratio in HQC-98.718 % and LQC-98.622 % -Simulated Serum

LONG TERM STABILITY

Performed in Human Serum at HQC and LQC level.

Mean Ratio in HQC-101.805 % and LQC-100.742 % -4 Week

Mean Ratio in HQC-109.353 % and LQC-102.895 % -Simulated Serum

NOVEL ASPECTS

Since colorimetric assays rely on acidic release of Iron from Transferrin, they are not very accurate as Iron-Carbohydrate complex is very stable in acidic conditions and they are also not capable of discriminating TBI from Total Iron.

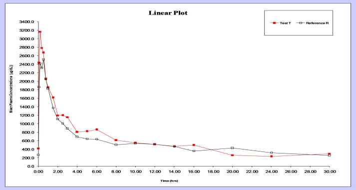
At Intervein, we developed and validated a robust and accurate method for analysis of Total Iron and Transferrin Bound Iron to generate pharmacokinetic data as a component of bioequivalence studies for generic intravenous preparation. We have developed accurate method to separate TBI using alumina basic columns and the final concentrations were calculated by subtracting TBI Concentration from Total Iron Concentration.

We have also compared the results of colorimetric assay and estimation by ICP-OES. The results is as below:

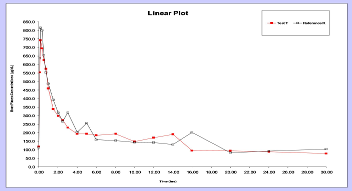
Pre-Dose		Iron		TBI	
Sr. No.	Time Point (min)	Colorimetry Result (µg/dL)	ICP-OES Result (µg/dL)	Colorimetry Result (µg/dL)	ICP-OES Result (µg/dL)
1	Zero hour samples	32.00	119.54	12.32	76.60
2		27.00	115.10	9.12	47.20
3		35.20	138.80	9.56	47.90
4		69.13	260.00	14.88	80.30
5		27.87	116.60	8.53	45.60

Post-Dose		Iron		TBI	
Sr. No.	Time Point (min)	Colorimetry Result (µg/dL)	ICP-OES Result (µg/dL)	Colorimetry Result (µg/dL)	ICP-OES Result (µg/dL)
1	5	3395.50	4740.90	340.75	2354.50
	10	5478.40	18203.10	157.31	9658.00
	20	2179.50	6031.60	112.10	3109.40
2	10	6014.60	21513.40	340.75	12137.50
	15	4188.10	10021.60	157.31	5848.90
	20	6301.00	7169.40	112.10	3880.60

The result of test and reference drug for total iron and Transferrin Bound Iron is as below:



Mean Linear Plot of Iron For Test & Reference Product



Mean Linear Plot of TBI For Test & Reference Product

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