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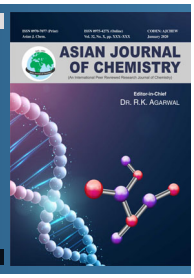

Analytical Methods for Ionic Profile of Dialysate Iron Therapy Drug Ferric Pyrophosphate Citrate By Ion Chromatography using Suppressed and Non-Suppressed Conductivity Detection

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In present study, ionic profile of pharmaceutical drug molecule ferric pyrophosphate citrate by ion exchange chromatography using conductivity detection was carried out. Ferric pyrophosphate citrate is an iron organic complex used for hemodialysis for the chronic kidney disease patients. The drug is used as iron supplementation to balance the iron loss during the dialysis for the kidney affected patients. Anions, cation and metal ion were analyzed by ion chromatographic method. Relevant anions sulphate, phosphate, citrate and pyrophosphate were analyzed by suppressed conductivity detection. Both isocratic and gradient separation methods were developed for the anions analysis. Cation sodium was analyzed by non-suppressed conductivity detection. Metal iron was determined by direct conductivity detection. All the analyses were done covering selectivity, precision, linearity and accuracy aspects of analysis. Calibration outcome of RSD 0.4 to 3.0% and correlation coefficient values greater than 0.999 were observed during the studies. Spiking studies were done to check the accuracy of the analysis and the recovery values ranging from 93 to 110% were observed. Around 90% ionic content of the drug molecule can be characterized using the developed ion chromatographic methods. These methods can be directly applied for the routine analysis of drug in the pharmaceutical industries.

Keywords: Ferric pyrophosphate, Chronic kidney disease, Hemodialysis, Iron supplementation, Ion exchange chromatography.

INTRODUCTION

Kidney disease is one of vital health problem throughout the worldwide. Kidney disease is defined as an abnormality of kidney structure or function with implications for the health of an individual, which can occur rapidly and either can be worked out or become chronic. There is an even higher prevalence of earlier stages of chronic kidney disease (CKD). It is a general term for heterogeneous disorders affecting kidney structure and function with variable clinical presentation, in part related to cause, severity and the rate of progression. Kidney failure is traditionally considered as the most serious outcome of CKD. Symptoms are usually due to complications of decreased kidney function and when severe, they can be treated only by dialysis or transplantation [1]. Hemodialysis or kidney dialysis, is a process of blood purification for a person whose kidneys are non-functional. During this blood purification process, waste products of blood such as urea and creatinine are removed from the blood. There will be a loss of iron for patients with hemodialysis dependent kidney disease. The iron loss happens due to iron requirements resulting from the use of erythropoiesis stimulating agents (ESAs) dialysis related blood losses including bleeding at gastrointestinal. Also the iron supply is suppressed by the chronic inflammation of renal failure, which stimulates hormone hepcidin. Hepcidin is the regulator of metabolism of iron and changed in response to anemia or inflammation. Serum ferritin and creatinine are the predictors for hepcidin levels in hemodialysis patients. Serum albumin and cholesterol are the predicting factors for hormone hepcidin levels in CKD patients [2].

Iron supplementation is necessary for the patients with CKD and hemodialysis to balance the loss of iron during the treatment [3]. To balance the iron losses, the hemodialysis patients are currently treated with iron supplementation either by intravenous (IV) iron or oral iron. Ferrous sulfate is one of the oral iron treatments between the meals, to the patients. Superior intravenous iron therapy is also common to the patients. Ferric gluconate and iron sucrose have become the predominant form

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of intravenous iron supplement to CKD patients [4,5]. Dialysate iron therapy is another way for iron supplementation. In this process, soluble form of iron is infused *via* the dialysate during the hemodialysis. Ferric pyrophosphate which is soluble is considered for dialysate iron therapy. In ferric pyrophosphate, pyrophosphate strongly complexes with iron and increases iron movement between serum ferritin and tissues during the dialysis [6,7]. Ferric pyrophosphate citrate is another iron complex, being recently tried as iron supplementation in dialysate therapy. This molecule is a sugar-free, soluble in water and iron complex used for dialysate iron therapy. During the hemodialysis, this molecule effectively replaces the iron losses, maintains the hemoglobin level without increasing the iron stores and displays protective way for iron maintenance [8-10]. Study outcome of this molecule is more encouraging in the hemodialysis related studies. Physical and chemical characteristics of iron complex are important information for the efficiency of hemodialysis applications. Major ion composition of this iron molecule is anions sulphate, phosphate, citrate, pyrophosphate, cation sodium and metal iron. This iron molecule is completely water soluble and analysis of the ionic composition is an important requirement for the dialysis applications.

Ion chromatography is an analytical technique for the separation and quantification of ions present in the sample matrix. Ion-exchange chromatography is based on a stoichiometric chemical reaction between ions in a solution and a normally solid substance carrying functional groups, which can fix ions as a result of electrostatic forces. In cation chromatography these are sulfonic acid or carboxyl groups, in anion chromatography quaternary ammonium groups. Since its introduction in 1975 [11], ion chromatography is developed and matured into an important analytical methodology in a number of diverse applications and industries, including pharmaceuticals [12]. In pharmaceutical industries, it is well accepted for inorganic anions and cations, amines, lanthanides, organic acids, carbohydrates, sugar alcohols, amino glycosides, amino acids, proteins, glycoprotein and potentially other analytes analysis [13-15].

This manuscript provides the information about the latest ion chromatography applications developed for ionic characterization of iron molecule ferric pyrophosphate citrate as per the current industry requirement. Four different ion chromatographic conditions were worked out for the characterization.

EXPERIMENTAL

High purity chemicals are required for ion chromatography analysis. Sodium carbonate, acetone, sodium hydroxide 50% w/v solution, nitric acid and acetonitrile used in these analyses were procured from Sigma-Aldrich, India. Certified ion standards of common anions, cations and iron were procured from Merck chemicals. Citric acid monohydrate and tetrasodium pyrophosphate were procured from Merck chemicals for citrate and pyrophosphate anions standards. Ultrapure water used in these studies was in complaint to ASTM, Type I water specification and the system of ELGA, PURELAB flex 3 is used for the purpose. Glassware′s eluent bottle, standard flasks of Borosil make were used for the developed methods. Micropipettes of Socorex brand were used for the standard preparations. Analytical balance of the model CPA225D from Sartorius was used to weigh chemicals for eluent, standard and sample preparations. Sample ferric pyrophosphate citrate for the study was obtained from one of the pharmaceutical industry, India.

Chromatographic system: Ion chromatograph instrument configuration was selected to meet the study requirements and nature of the sample matrix. Professional Ion chromatograph system with model 940 Professional IC from M/s. Metrohm, AG (Herisau, Switzerland) was used for all these studies. Suppressor MSM was used for suppressed conductivity detection for anions analysis. Professional Conductivity detector with Digital Signal processing technology and thermostated @ 40 ºC was used for the analysis. Automation system 858 Professional Sample Processor was used for the analysis. Data acquisition was done with software MagICnet, which is fully compliant to 21 CFR part 11 requirements.

Separation columns: Metrosep A Supp 4 250/4.0, Metrosep A Supp 3 250/4.6 and Metrosep A Supp 4/5 Guard/4.0 were used for suppressed conductivity anions analysis. Analytical separation column Metrosep C4 150/4.0 and Guard column Metrosep C4 Guard/4.0 were selected and used for sodium analysis by non-suppressed conductivity detection. Non suppressed direct conductivity detection was selected for iron analysis using the analytical column Nucleosil 5SA 125/4.0 along with Guard column Nucleosil 5SA 2 Guard Cartridge/4.0.

Analytical methods developed:

1) Determination of anions sulphate, phosphate, citrate and pyrophosphate was done using 4.0 mM sodium carbonate and 15 mM sodium hydroxide + 10% acetonitrile in ultrapure water as eluent. Separation was carried out using the single composition eluent at room temperature.

Sample preparation: Sample ferric pyrophosphate citrate is completely water soluble. Sample concentration of 100 mg/L in ultrapure water was prepared for the analysis. Around 50 mg of sample was accurately weighed in a 50 mL standard measuring flask, to which ultrapure water was added up to the mark. The sample solution was sonicated for 5 min and 1 mL of sample solution was further diluted to 10 mL using ultrapure water. The diluted sample solution was filtered and injected into ion chromatograph. Sample loop size of 20 µL was used for the analysis.

e) Determination of anions sulphate, phosphate, citrate and pyrophosphate was done using binary gradient separation using the eluents ultrapure water and 50 mM NaOH. Separation was carried out using the combination of step and linear gradient of both the eluents at room temperature. Same sample preparation was followed for the analysis. Sample loop size of 20 µL was used for the analysis.

Sample preparation: The samples were prepared as same above.

3) Determination of sodium was done using 2.0 mM nitric acid in ultrapure water as eluent. The separation was carried out with stationary phase Metrosep C4 150/4.0 using isocratic separation by non-suppressed conductivity detection

Sample preparation: Sample concentration of 25 mg/L in ultrapure water was prepared for the analysis. Around 25 mg of sample was accurately weighed in a 50 mL standard flask, to which ultrapure water was added up to the mark. The sample

solution was sonicated for 5 min and 0.5 mL of sample solution was further diluted to 10 mL using ultrapure water. The diluted sample solution was filtered and injected into ion chromatograph. Sample loop size of 5 µL was used for the analysis.

4) Determination of iron was done using 4.0 mM tartaric acid, 0.5 mM citric acid and 3.0 mM ethylene diamine + 5% acetone in ultrapure water as eluent using non-suppressed conductivity detection. Diluent: 1000 mg/L ascorbic acid in ultrapure water.

Sample preparation: Sample concentration of 25 mg/L in diluent was prepared for the analysis. Around 25 mg of sample was accurately weighed in a 50 mL standard measuring flask, to which diluent was added up to the mark. The sample solution was sonicated for 5 min and 0.5 mL of the sample solution was further diluted to 10 mL using diluent. The diluted sample solution was filtered and injected into ion chromatograph. Sample loop size of 5 µL was used for the analysis. Diluent was injected as blank and iron was not detected.

RESULTS AND DISCUSSION

In the present study, four ion chromatographic separation methods were worked out for the drug. Isocratic and gradient separation methodologies for applicable anions sulphate, phosphate, citrate and pyrophosphate quantification. Additionally, two other methods for cation sodium and iron quantification in the drug. All these methods were worked out as per the pharmaceutical industries method development requirements. The chromatographic conditions were tested for selectivity, linearity and accuracy. Linear calibration curves were obtained with certified standards and correlation coefficient greater than 0.999 was achieved for all the analysis. The obtained calibration parameters for all the methods are listed in Table-1. Accuracy of the method was checked by spiking the sample with ionic standards and the recovery values were in the range of 93.0 to 110%. These were simple, straight forward ion chromatography methods and easily adoptable for regular pharmaceutical quality control/research applications.

Analysis of anions by isocratic separation (analytical method 1): Ferric pyrophosphate citrate contains sulphate, phosphate, citrate and pyrophosphate. Among these anions, sulphate is divalent, phosphate and citrate are trivalent in the selected chromatographic condition and pyrophosphate is tetravalent anion. Simple separation methodology was worked out for the quantification of these applicable anions by suppressed conductivity detection. Single composition eluent containing 15 mM NaOH, 4 mM Na_2CO_3 + 10% acetonitrile was selected after optimization for the separation and analysis. Separation column with poly(vinyl alcohol) as base material functionalized with quaternary ammonium ion group was selected. Strong eluting ion carbonate was added to the eluent to get earlier elution of all anions. Sodium hydroxide was added at 15 mM to attain the eluent as alkaline and to ensure that ions in the ionic stage during the separation. Phosphate will be trivalent when more than 3 mM NaOH present in the eluent. In this selected condition, all the other common monovalent anions were found to be eluting before sulphate elution. Organic modifier acetonitrile (10%) was added to improve the peak shape and also to keep column clean after the sample analysis. Elevated temperature separation was tried and found that the peak shape of pyrophosphate was disturbed and tailing. Hence, the analysis was done at room temperature. Flow rate of eluent was kept at 1 mL/min till the phosphate elution and then kept at 1.5 mL/min for the earlier elution of citrate and pyrophosphate. Specificity of the analytes were checked with 1 mg/L mixed anions standard are reported in Table-2. Specificity chromatogram of this chromatographic condition is provided in Fig. 1. Packed bed suppressor, MSM was used for the analysis. As per the manufacturer instruction, the MSM was regenerated with regeneration solution and rinsed with ultrapure water. Generally, 100 mM sulphuric acid will be the regeneration solution. But, this analysis was with molecule containing iron, hence, regeneration solution of 100 mM sulphuric acid along with 20 mM oxalic acid was used. Alternatively, 100 mM phosphoric acid is also recommended. In this analysis, the disturbance at 13th min was due to suppressor step given in the time programme and ensured that this was not affecting the analysis. The analysis was carried out with reasonable run time of 25 min. Six points calibration ranging from 1 to 30 mg/L mixed standards and sample

TABLE-1
CALIBRATION PARAMETERS FOR ALL THE FOUR IC METHODOLOGIES
Method 1: Anions sulphate, phosphate, citrate and pyrophosphate analysis by suppressed conductivity detection using isocratic separation.
Method 2: Anions sulphate, phosphate, citrate and pyrophosphate analysis by suppressed conductivity detection using gradient separation.
Method 3: Cation sodium analysis by non-suppressed conductivity detection using isocratic separation.
Method 4: Metal iron analysis by non-suppressed conductivity detection using isocratic separation.

Method	Analytes	Capacity factor/ specificity	Calibration range	Function	Correlation coefficient	RSD (%)	Accuracy/ Recovery (%)
Method 1 (isocratic)	Sulphate	4.35	1 to 30 mg/L	A = -0.14856 + 9.9088E-3xQ	0.9996	2.5	109
	Phosphate	5.99		A = -0.010042 + 3.55752E-3xQ	0.9997	1.9	110
	Citrate	9.40		A = -0.01426 + 1.28848E-3xQ	0.9999	0.9	99
	Pyrophosphate	17.22		A = -7.2597E-3 + 1.9902E-3xQ	0.9997	1.9	103
Method 2 (gradient)	Sulphate	9.60	1 to 30 mg/L	A = 0.06917 + 0.01253xQ	0.9999	0.4	100
	Phosphate	10.72		A = 0.06960 + 5.6308E-3xQ	0.9998	1.6	95
	Citrate	12.23		A = 0.07912 + 4.46867E-3xQ	0.9993	2.9	93
	Pyrophosphate	15.45		A = -0.11785 + 5.20769E-3xQ	0.9994	3.0	105
Method 3 (sodium analysis)	Sodium	5.72	0.1 to 10 mg/L	A = 0.0147526 + 0.0363045× Q	0.9999	1.7	98
Method 4 (iron analysis)	Iron	6.76	0.25 to 10 mg/L	A = 7.39933E-4 + 3.78746E-3× Q	0.9999	1.5	107

TABLE-2
SPECIFICITY RESULTS

Anions	Retention time for Isocratic method	Retention time for Gradient method
Fluoride	2.73	6.59
Chloride	3.17	7.18
Nitrite	3.36	7.66
Bromide	3.87	8.07
Nitrate	3.87	8.58
Sulfate	4.35	9.60
Phosphate	5.99	10.72
Citrate	9.40	12.23
Pyrophosphate	17.22	15.45

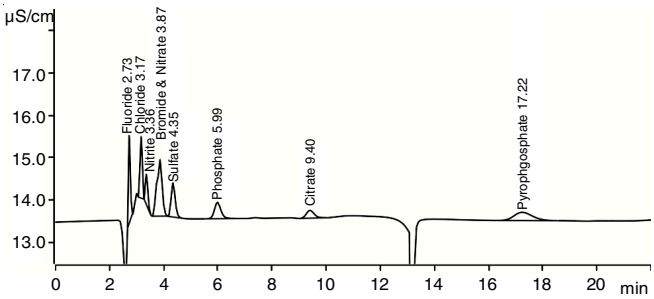

Fig. 1. Specificity chromatogram for mixed anions standard comprises of 1 mg/Leach of common anions and 5 mg/L each of citrate, pyrophosphate; isocratic separation; Method 1

analysis were carried out. Calibration outcome is reported in Table-1. Chromatograms for different concentrations are shown in Fig. 2. Sample was prepared at 100 mg/L concentration and analyzed. Repeatable results of sulphate 24.8%, phosphate 0.5%, citrate 15.7% and pyrophosphate 18.4% were determined in the sample. The sample chromatogram is shown in Fig. 3 and the sample results are reported in Table-3. Accuracy of the analysis by spiking mixed anions containing 20 mg/L each of sulphate, citrate and pyrophosphate and 1 mg/L phosphate standard with sample was done. Recovery values were calculated using the area values and found to be in the range of 99 to 110%. All the data are given in Table-1.

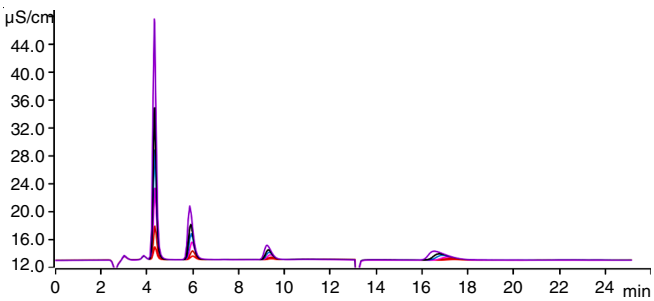

Fig. 2. Linearity of mixed anions from 1 to 30 mg/L for isocratic separation condition; Method 1

TABLE-3
SAMPLE RESULTS (%)

Analytes	Isocratic method	Gradient method
Sulfate	24.8	24.4
Phosphate	0.5	0.4
Citrate	15.7	15.9
Pyrophosphate	18.4	18.5
Sodium	18.1	N/A
Iron	7.8	N/A

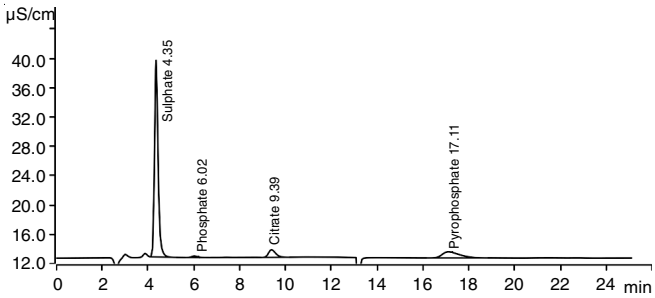

Fig. 3. Chromatogram for sample analysis for anions estimation using the isocratic separation condition; Method 1

Analysis of anions by gradient separation (Analytical method 2): This chromatographic condition was developed to see the effect of gradient separation and peak shape of late eluting anions pyrophosphate and citrate. This condition was done with two eluents, eluent A was ultrapure water and eluent B was 50 mM NaOH. The gradient programme was selected after optimization and its details are given in Table-4. Initial stage of separation was with more eluent A and later stage of separation with more eluent B during the analysis. Separation condition was optimized and selectivity was ensured for applicable four anions in the drug from common anions. Specificity of the analytes were checked with 10 mg/L mixed anions standard are reported in Table-2 and the specificity chromatogram is shown in Fig. 4. Packed bed suppressor MSM-HC high capacity was used for the analysis. There was no need to give step command during the analysis. Oxalic acid (20 mM) was used along with sulphuric acid for regeneration of suppressor. Alternatively, 100 mM phosphoric acid is also recommended. It was observed that the responses for citrate and pyrophosphate anions were higher compared to isocratic method. It was due to more ionization of these anions in more alkaline condition using higher concentration NaOH as eluent. Using this gradient method, six points calibration was done with mixed standards ranging from 1 mg/L to 30 mg/L. Calibration outcomes are reported in Table-1 and the linearity study chromatograms are shown in Fig. 5. Sample concentration of 100 mg/L was prepared and analyzed. Repeatable results of sulphate 24.4%, phosphate 0.4%, citrate 15.9% and pyrophosphate 18.5% were found. The sample results are reported in Table-3 and the sample chromatogram is shown in Fig. 6. Analysis accuracy was checked by spiking mixed anions containing 20 mg/L each of sulphate, citrate and pyrophosphate and 1 mg/L phosphate standard with sample. Recovery values were calculated using the area values and found to be in the range of 93 to 105%.

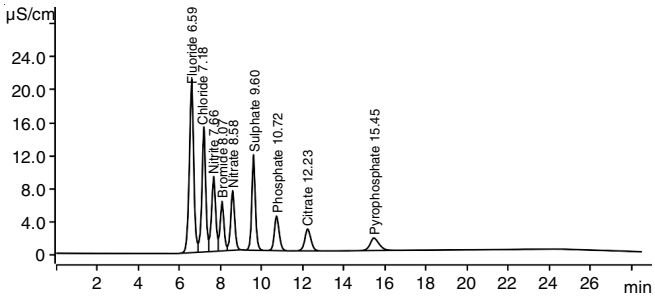

Fig. 4. Specificity chromatogram for 10 mg/L mixed anions standard comprises of common anions, citrate and pyrophosphate by gradient separation condition; Method 2

TABLE-4
PROGRAMME FOR GRADIENT SEPARATION

Time	% A (Water)	% B (50 mM NaOH)
0	95	5
4	90	10
4.5	68	32
20	15	85
26	95	5

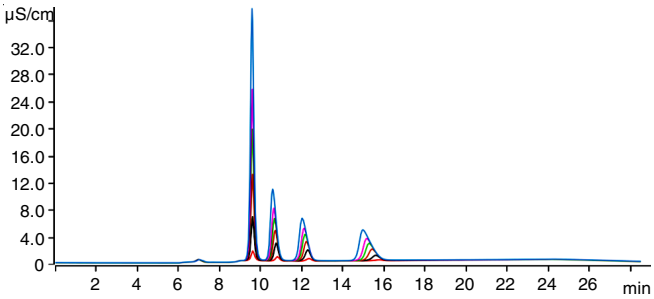

Fig. 5. Linearity of mixed anions from 1 to 30 mg/L for gradient separation condition; Method 2

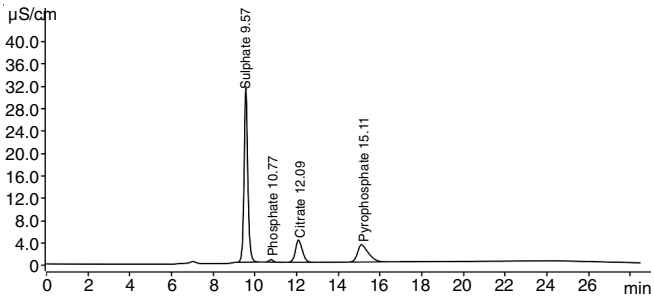

Fig. 6. Chromatogram for sample analysis for anions estimation using the gradient separation condition; Method 2

Analysis of cation sodium in drug by non-suppressed conductivity detection (Analytical method 3): The drug molecule was also tested for its applicable sodium ion by non-suppressed conductivity detection. Eluent concentration of 2.0 mM nitric acid was selected and the selectivity of sodium with other common cations was ensured. Linearity with six point's calibration ranging from 0.1 to 10 mg/L was carried out. Outcomes of linearity study are given in Table-1 and the sample chromatogram is shown in Fig. 7. Sample concentration of 25 mg/L was prepared and analyzed. Sodium ion concentration of 18.1% was found in the drug molecule and reported in Table-3. Accuracy study was done and the recovery value was found to be 98%.

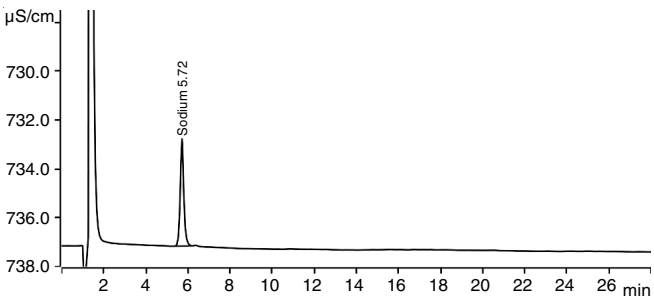

Fig. 7. Chromatogram for sample analysis for sodium estimation using non-suppressed conductivity detection. Eluent: 2 mM nitric acid; Method 3

Analysis of metal iron in drug by direct conductivity detection (Analytical method 4): Iron content present in the drug molecule was determined with cation exchange column Nucleosil 5SA by direct conductivity detection. In the selected chromatographic condition, iron was determined as Fe^{2+}. Diluent for the analysis was ascorbic acid with a concentration of 1 mg/mL. Reducing agent ascorbic acid diluent was selected to convert all iron to Fe^{2+} state. Ascorbic acid 2 mg/L was prepared in ultrapure water, analyzed as diluent and iron was not detected. Standard and samples were prepared with diluent. Selectivity for iron with other metals copper, nickel, zinc, manganese, cadmium, cobalt and divalent cations calcium and magnesium was ensured. Linearity for six point's calibration ranging from 0.25 to 10 mg/L was performed and the results are shown in Table-1. Sample concentration of 25 mg/L was prepared in the diluent and analyzed. Iron concentration of 7.8% was determined and reported in Table-3 and the sample chromatogram is shown in Fig. 8. Accuracy study was done by spiking iron standard with sample and recovery value of 107% was observed. Iron content in the sample was analyzed with voltammetry technique using 797 VA computrace, Metrohm, by standard addition method and the iron content of 8% was found. Thus, voltammetry result was found to be conforming to the findings of ion chromatography technique.

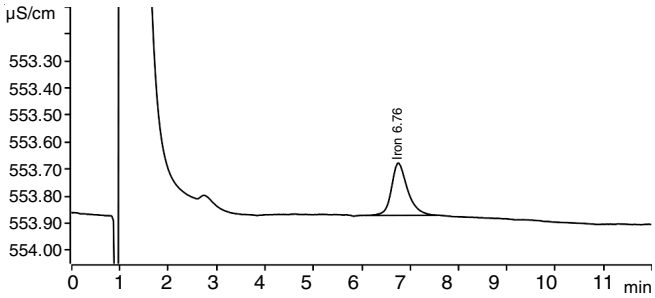

Fig. 8. Chromatogram for sample analysis for Iron estimation using direct conductivity detection; Method 4

Conclusion

In this study, both isocratic and gradient separation conditions were worked out for quantification of applicable anions sulphate, phosphate, citrate and pyrophosphate using anion exchange chromatography by suppressed conductivity detection. Findings of sample analysis by both the separation conditions were found to be comparable and discussed in this paper. In the drug, relevant cation sodium was analyzed by cation exchange chromatography using non-suppressed conductivity detection. Iron ion was also analyzed using cation exchange chromatography by direct conductivity detection. The developed analytical techniques with ion chromatograph and conductivity detector offers simple and straight-forward methods for the analysis of all relevant ions *viz*. anions, cation and metal ion in ferric pyrophosphate citrate. The presented single analytical technique ion chromatography with three methods can be used to characterize around 90% of various ions present in the drug. Both isocratic and gradient separation methods were presented for the analyst to choose and work based on the instrument configuration available. These methods can be directly employed for the routine ionic characterization of drug in pharmaceutical industries.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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