Assay for Citrate and Phosphate in Pharmaceutical Formulations Using a High-Pressure Compact Ion Chromatography System

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Key Words
Small particle column, Microbore, Integrion, HPIC, Fast IC, USP method

Goal

To update Application Note 164 with fast separations using a Thermo Scientific™ Dionex™ Integrion™ HPIC™ system and a Thermo Scientific™ Dionex™ IonPac™ AS11-HC-4µm microbore column.

Introduction

Citric acid is an ingredient of many pharmaceutical formulations.[1] It provides an effervescent effect when combined with carbonates or bicarbonates in antacids and dentifrices. Citrate is added as a flavoring and stabilizing agent in some pharmaceutical products to mask the taste of medicinal flavors or as a buffering agent to maintain stability of the active pharmaceutical ingredient (API) and improve the effectiveness of an antioxidant. Additionally, citrate has been used in anticoagulants to preserve blood and to prevent excess bleeding during rectal enema treatments.

As the chromatographic technique of choice for citrate determinations, ion chromatography (IC) with suppressed conductivity detection has been validated in AN164[1,2] and featured in the United States Pharmacopeia (USP) General Chapter <345>, Assay for Citric Acid/Citrate and Phosphate.[3] The method for citric acid determination was first published in the official 2006 edition of the United States Pharmacopeia and National Formulary (USP 29–NF 24). In this method, citrate and phosphate are separated on a L61 column (a Dionex IonPac AS11 hydroxide-selective anion exchange column), using 20 mM NaOH or KOH at a 2 mL/min flow rate. Both phosphate and citrate are eluted from the column and determined within 10 minutes.

Ion chromatography technology has advanced greatly in the past 10 years. A recently introduced compact IC system, the Thermo Scientific™ Dionex™ Integrion™ HPIC™ System, includes many advances in IC instrument technology, such as high pressure capabilities for Reagent-Free™ IC (RFIC™) (up to 5000 psi), column heater control, and other new features designed to increase customer ease-of-use. These include:

- Simplified plumbing layout with easy-to-install Thermo Scientific™ Dionex™ IC PEEK Viper™ fittings connections in key positions to minimize void volume problems.
- Separate compartments for pump, column heater with injection valve, and detection-suppressor to provide separate temperature control and faster equilibration.
- Components tracked by consumable device tracking technologies for better GMP compliance tracking and to assure installation of compatible devices (i.e., installation of non-compatible devices is prevented).
- Detachable tablet for convenient IC control and continuous, full-screen monitoring. The tablet also provides the online instrument manual and trouble-shooting guides. All are available in local language.
- New Thermo Scientific™ Dionex™ Chromeleon 7 Chromatography Data System (CDS) software features, providing easy instrument configuration, monitoring of consumable devices, and online video instructions for conditioning columns, suppressors, and other electrolytic devices.

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Previously, it was shown that this analysis can be run successfully on the Dionex Integrion HPIC system.[4] Here, we update the method described in AN164 for citrate and phosphate determinations using the high-capacity Dionex IonPac AS11-HC-4µm column, which has similar selectivity to L61, on the Dionex Integrion HPIC system. This method demonstrates reduced run times from 10 to 5 min. Following the guidelines outlined in USP General Chapter <1225>, Validation of Compendial Methods[5], the improved IC method is evaluated in terms of linearity, precision, accuracy, robustness, and limit of quantitation (LOQ).

Equipment

Thermo Scientific™ Dionex™ Integrion™ HPIC™ system including:

- The Dionex Integrion HPIC system pump
- CD Conductivity Detector
- Detector compartment temperature control
- Column oven temperature control
- Tablet control
- Consumables device tracking capability
- Eluent generation

Thermo Scientific™ Dionex™ AS-AP Autosampler with 10 mL trays

Table 1 lists the consumable products needed for the Dionex Integrion HPIC system, configured for suppressed conductivity detection.

Software

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data system (CDS) software CM 7.2 SR4.

Reagents and Standards

- Deionized water, 18 MΩ-cm resistivity or better
- Citric acid (USP, Catalog #1134368)
- Sodium dihydrogen phosphate monohydrate, $NaH_2PO_4 \bullet H_2O$ (EM Science)
- Sodium Hydroxide Solution, 50% (w/w), aqueous NaOH (Fisher Scientific)

It is important to use 18 MΩ-cm resistivity, deionized water (DI) for standards, eluent, and autosampler flush solutions to avoid system contamination, decreased sensitivity, and poor calibration. Degassing the DI water by vacuum filtration prior to use is a good practice.

Table 1. Consumables list for the Dionex Integrion HPIC system.

Product name	Product Description	Part Number
Thermo Scientific™ Dionex™ IC PEEK Viper™ fitting tubing assembly kits	Dionex IC Viper fitting assembly kit for the Integrion: Includes one each of P/Ns: 088805–088811	088798
Dionex IC PEEK Viper fitting tubing assemblies	Guard to separator column: 0.007 in i.d., 4.0 in long (102 mm)	088805
	Injection Valve, Port C (Port 2) to guard column: 0.007 in i.d., 5.5 in long (140 mm)	088806
	EGC Eluent Out to CR-TC Eluent In: 0.007 in i.d., 6.5 in long (165 mm)	088807
	Separator column to Suppressor Eluent In: 0.007 in i.d., 7.0 in (178 mm)	088808
	Suppressor Eluent Out to CD In: 0.007 in i.d., 9.0 in long (229 mm)	088810
	CR-TC Eluent Out to Degasser Eluent In: 0.007 in i.d., 9.5 in long (241 mm)	088811
Dionex AS-AP Autosampler vials	Package of 100, polystyrene vials, caps, blue septa,10 mL	074228
Thermo Scientific™ Dionex™ EGC 500 KOH Potassium Hydroxide Eluent Generator Cartridge	Eluent generator cartridge	075778
Thermo Scientific™ Dionex™ CR-ATC 600 Continuously Regenerated Anion Trap Column	Continuously regenerated trap column used with Dionex EGC 500 KOH cartridge	088662
HP EG Degasser Module	Degasser installed after Dionex CR-TC trap column and before the injection valve. Used with eluent generation.	075522
Thermo Scientific™ Dionex™ AERS™ 500 Anion Electrolytically Regenerated Suppressor	Suppressor for 2 mm columns, using recycle mode	082541
Dionex IonPac AG11-HC-4µm Guard Column	Anion guard column, 2 × 50 mm	078036
Dionex IonPac AS11-HC-4µm Analytical Column	Anion analytical column, 2 × 250 mm	078035
Thermo Scientific™ Nalgene™ syringe filter	Syringe filters, 25 mm, PES membrane, 0.2 µm. This type is compatible with IC analysis.	Thermo Scientific 725-2520*

* Fisher Scientific P/N 09-740-113

20 mM NaOH Matrix Solution

Pipet 1.05 mL of 50% (w/w) aqueous NaOH from the reagent bottle into a 1.00 L volumetric flask containing about 500 mL of degassed DI water. Bring to volume with degassed DI water. Prepare daily.

Stock Standard Solutions

To prepare a 1000 mg/L citrate stock standard, weigh 100 ± 0.1 mg of the citrate (as 101.6 mg of freshly opened official USP citric acid reference standard), add to a 100 mL volumetric flask. Add ~ 25 mL of DI water, swirl to dissolve the citrate reagent and dilute to volume with DI water. To prepare a 600 mg/L phosphate stock standard, weigh 60 ± 0.1 mg of phosphate (as 87.16 mg of $NaH_2PO_4 \bullet H_2O$), add to 100 mL volumetric flask. Dissolve with ~25 mL DI water, and dilute to volume with DI water. To prepare a 500 mg/L citrate and 300 mg/L phosphate mixed citrate/phosphate stock standard, add equal parts of the individual citrate and phosphate stock standards. Store all stock standards in polypropylene bottles at 4 °C.

Working Standard Solutions

To prepare working standard solutions in 1 mM NaOH, add an appropriate amount from the stock standard solutions, 5 mL of 20 mM NaOH and DI water. The 20 mM NaOH solution used for standard and sample preparation should be prepared fresh daily. The mixed standard containing 20 mg/L citrate and 12 mg/L phosphate is used to test the robustness of the assay for citrate and phosphate in samples.

Sample

Anticoagulant Citrate Phosphate Dextrose Solution (CPD) was purchased from Novateinbio (Cat# NIBB-410). According to Novateinbio, the CPD solution contains 90 mM trisodium citrate dihydrate, 17 mM citric acid, 18.5 mM monobasic sodium phosphate, and 142 mM dextrose.

The sample was diluted 100-fold for phosphate and 1000-fold for citrate determinations with 1 mM NaOH so that the concentration of citrate and phosphate were within the calibration ranges.

Chromatographic Conditions for the Assay

Columns	Dionex IonPac AG11-HC-4µm guard (2 × 50 mm) and Dionex IonPac AS11-HC-4µm analytical (2 × 250 mm)
Eluent	60 mM KOH
Eluent Source	Dionex EGC 500 KOH cartridge with Dionex CR-ATC 600 trap column and high pressure EG degasser
Flow Rate	0.35 mL/min
Column Temp	35 °C
Detector Compartment Temperature	20 °C
Injection Volume	2.5 µL, in Push Full mode
Detection	Suppressed conductivity, Dionex AERS 500 suppressor, 2 mm, recycle mode, 52 mA
Run time (min)	5
Background Conductance (µS)	< 1
Typical Noise (nS)	< 1
System backpressure (psi)	~ 4000

Instrument Setup and Installation

The Dionex Integrion HPIC system is a high-pressure-capable, integrated RFIC system. The Dionex Integrion HPIC system and the Dionex EGC 500 KOH cartridge and Dionex CR-ATC 600 trap column consumable products are designed for high pressure conditions up to 5000 psi.

To set up this application, connect the Dionex AS-AP autosampler and the Dionex Integrion HPIC system as shown in Figure 1.

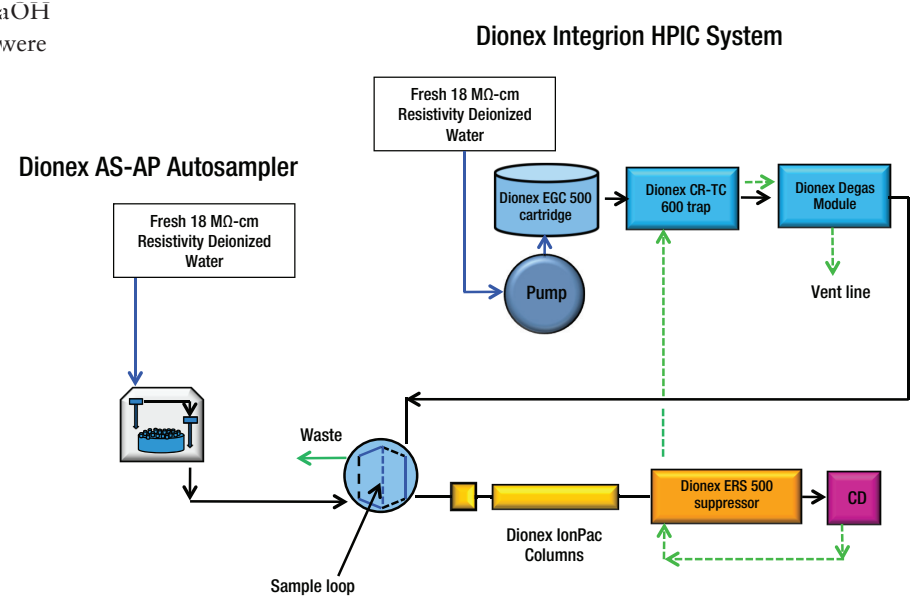


Figure 1. Flow diagram for the Dionex Integrion HPIC system.

Connect the USB cables from the Dionex Integrion HPIC system to the Dionex AS-AP autosampler and to the computer. Connect the power cables and turn on the IC instrument and the autosampler.

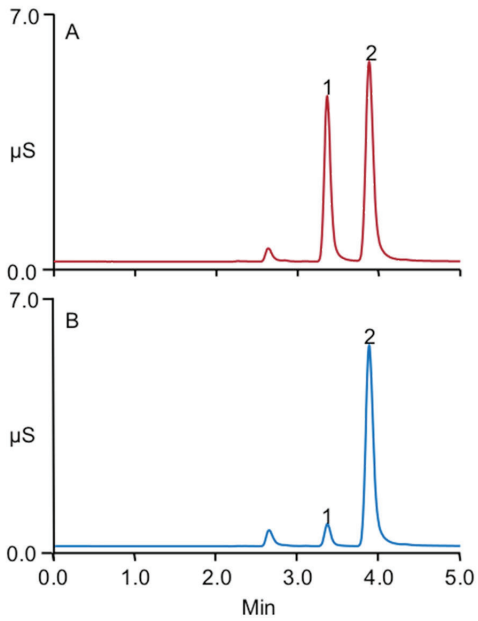
The following are important steps to prepare the system for the analysis. Details can be found in AU 200[6] or TN 175.[7]

- Configuring the modules in the Chromeleon CDS software
- Plumbing the high pressure Dionex Integrion HPIC system
- Conditioning electrolytic devices and columns
- Installing and optimizing the Dionex AS-AP autosampler
- Starting the Dionex Integrion HPIC system
- Creating an instrument method

Results

The Dionex Integrion HPIC system was designed to run analyses with eluent generation up to 5000 psi. An improved IC method for the assay for citric acid/citrate and phosphate was developed using a Dionex Integrion HPIC system and a 2 mm Dionex IonPac AS11-HC-4µm column set. The Dionex IonPac AS11-HC-4µm columns have smaller (4.0 vs. 13 µm) and higher porosity (pore size 2000 Å vs. <10 Å) resin particles with the same functional groups as the L61 column. As a result, the Dionex IonPac AS11-HC-4µm column has similar chromatographic selectivity as L61 combined with higher capacity and efficiency than an L61 column. The methods run on the L61, Dionex IonPac AS11 column can be easily transferred to the Dionex IonPac AS11-HC-4µm column with the benefit of increased peak resolution and faster analysis time. The microbore format provides reduced eluent consumption, which reduces operating costs.

Figure 2 shows the separation of phosphate and citrate on a 2 mm Dionex IonPac AS11-HC-4µm column in a (A) standard and (B) in an anticoagulant citrate, phosphate, dextrose, and adenine dosage form. Using an electrolytically generated 60 mM hydroxide eluent, phosphate and citrate were well separated in 5 min, saving 5 min compared to the separation time reported in AN164. Additionally, the lower flow rate (0.35 mL/min over the previous 2 mL/min) extends the lifetime of the Dionex EGC 500 KOH cartridge. Overall, each separation requires only 1.75 mL of eluent compared to 20 mL in the original application.



Columns:	Dionex IonPac AG11-HC-4µm Dionex IonPac AS11-HC-4µm, 2 mm
Eluent:	60 mM KOH
Eluent Source:	Dionex EGC-500 KOH cartridge, with Dionex CR-ATC 600 trap column, Dionex high pressure degasser
Flow Rate:	0.35 mL/min
Inj. Volume:	2.5 µL
Column Temp.:	35 °C
Detection:	Suppressed conductivity, Dionex AERS 500, 2 mm, 20 °C, 52 mA, recycle mode
Sample :	A: Mixed standard B: CPD Solution, 1000-fold diluted. (Anticoagulant Citrate Phosphate Dextrose)

Peaks:	A	B	
1. Phosphate	12	1.6	mg/L
2. Citrate	20	20	

Figure 2. Separation of phosphate and citrate on a 2mm Dionex IonPac AS11-HC-4µm column. (A) Standard. (B) Anticoagulant citrate, phosphate, dextrose, and adenine dosage form.

The LOQs were determined following the guidelines outlined in USP General Chapter <1225>, Validation of Compendial Methods.[3] The noise is calculated using eight injections of the lowest calibration standard 0.5 mg/L, and LOQ = 10 × δ (standard deviation of concentration by peak area). Table 2 summarizes the calibration and LOQs for citrate and phosphate. This method has lower LOQs with 25% the injection volume of the original method (phosphate 0.03 vs. 0.2 mg/L and citrate 0.06 vs. 0.2 mg/L). Over the calibration range of 0.5 to 200 mg/L for phosphate and 0.5 to 50 mg/L for citrate, the calibrations are linear. When the concentration range is extended above 50 mg/L, the peak area versus concentration relationship for citrate is best fit by a quadratic equation.

The method performance was measured by determining the precision of replicate sample injections and recovery of spiked samples. The relative standard deviations (RSDs) were calculated for samples prepared with the target analytes at a concentration of ~20 mg/L citrate and 12 mg/L phosphate. The intraday precisions for citrate and phosphate from independently prepared solutions analyzed on separate days were < 0.18–0.34% for phosphate and < 0.05–0.57% for citrate. The interday precisions over the four day period were 0.75% for phosphate and 1.25% for citrate. Recoveries were determined by adding known amounts of analyte to the sample solutions. The calculated recoveries were from 95.2–105.5% for all samples.

Table 3 summarizes the precision and recovery results for citrate and phosphate. The precision ranges are for each of the four days, using three independently prepared solutions each day. The spike recoveries are from spiking 2.0 mg/L of citrate or phosphate into the samples.

Table 2. Summary of calibration and limit of quantitation data for citrate and phosphate.

	Calibration Range (mg/L)	Calibration Type	Coefficient of Determination (r^2)	LOQ (mg/L)
Phosphate	0.5–200	Linear	1.0000	0.03
Citrate	0.5–200	Quadratic	0.9998	
Citrate	0.5–50	Linear	0.9997	0.06

Table 3. Accuracy and precision for citrate and phosphate in the pharmaceutical formulation.

	Intraday Precision Ranges (RSD)	Interday Precision (RSD)	Range of Recoveries (%)
Phosphate	0.18–0.34	0.75	95.6–105.5
Citrate	0.05–0.57	1.25	95.2–98.1

Table 4 compares the measured citrate and phosphate concentrations in the CPD solution to the amounts listed on the label and USP monographs.[8] The measured value was very close to the label amounts for citrate but about 6% different for phosphate. The label amount of phosphate appears incorrect. This hypothesis was supported when comparing the measured values to the USP specification. The label amount of phosphate is out of specification. The measured values were within the USP specification for both citrate and phosphate.

The USP defines robustness of an analytical method as a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage. Using the USP guidelines for chromatography, the robustness of this method was evaluated by examining the retention time (RT), peak asymmetry, and resolution of the two analytes in the mixed standard containing 20 mg/L of citrate and 12 mg/L of phosphate after imposing small variations (± 10%) in procedural parameters. (e.g., flow rate, eluent gradient concentration, and column temperature). Due to the maximum flow rate allowed for this column, a 0.380 mL/min flow rate was used instead of the 0.385 mL/min flow rate used for the faster flow test. Test results for different variations are summarized in Table 5. Peak asymmetry was calculated using the USP formula. The peak asymmetries were similar for all conditions. As expected, the reduced temperature (31.5 vs. 35.0 °C) and higher eluent concentration (66 vs. 60 mM KOH) decrease the retention time and resolution. In the worst case, at the highest eluent concentration and lowest

Table 4. Comparison of the citrate and phosphate concentrations obtained to the label amounts and USP Monograph.

	Label Amount (mg/mL)	USP Spec. (mg/mL)	Experimental Average ± standard deviation (mg/mL)
Phosphate	1.75	1.50–1.65	1.65 ± 0.01
Citrate	20.2	19.16–21.18	20.15 ± 0.04

Table 5. Robustness of the assay for citrate and phosphate. *

Parameter		Phosphate RT. (min)	Diff. (%)	Asym.	Diff. (%)	Resol.	Diff. (%)
Flow rate (mL/min)	0.315	3.72	10.68	1.31	1.35	3.28	0.54
	0.35	3.36		1.30		3.26	
	0.38	3.12	-7.20	1.27	-1.74	3.28	0.54
Column Temp. (°C)	31.5	3.19	-5.19	1.30	0.00	2.63	-19.34
	35.0	3.36		1.30		3.26	
	38.5	3.55	5.46	1.29	-0.19	3.96	21.49
Eluent Conc. (mM KOH)	54	3.85	14.47	1.27	-1.74	4.50	37.99
	60	3.36		1.30		3.26	
	66	3.05	-9.40	1.31	1.16	2.40	-26.25
Parameter		**Citrate RT. (min)**	**Diff. (%)**	**Asym.**	**Diff. (%)**	**Resol.**	**Diff. (%)**
Flow rate (mL/min)	0.315	4.29	10.65	1.36	-0.18		
	0.35	3.88		1.36			
	0.38	3.60	-7.10	1.38	1.65		
Column Temp. (°C)	31.5	3.59	-7.44	1.37	0.55		
	35.0	3.88		1.36			
	38.5	4.20	8.17	1.35	-0.74		
Eluent Conc. (mM KOH)	54	4.64	19.65	1.31	-3.49		
	60	3.88		1.36			
	66	3.40	-12.34	1.42	4.04		

*Average of three injections of the 12 mg/L phosphate and 20 mg/L citrate mixed standard

Table 6. Robustness of the assay for citrate and phosphate in a pharmaceutical formulation.*

Parameter		Phosphate (mg/L)	Diff. (%)	Citrate (mg/L)	Diff. (%)
Flow rate (mL/min)	0.315	16.55	0.30	20.23	0.39
	0.35	16.50		20.15	
	0.38	16.58	0.46	20.19	0.21
Column Temp. (°C)	31.5	16.44	-0.37	20.26	0.55
	35.0	16.50		20.15	
	38.5	16.57	0.42	20.20	0.24
Eluent Conc. (mM KOH)	54	16.58	0.47	20.17	0.13
	60	16.50		20.15	
	66	16.19	-1.88	20.19	0.21

* Average of three injections of phosphate in 100-fold diluted CPD solution and citrate in 1000-fold diluted CPD solution.

temperature, citrate and phosphate retained baseline resolution of > 1.5. In addition, a 10% shift in eluent concentration or temperature is unlikely to occur on a Dionex Integrion HPIC system as a result of its eluent generation capabilities and the temperature control in the column and detector compartments. The robustness of this method was also evaluated by comparing the amount of citrate and phosphate measured at these conditions for the samples. Table 6 summarizes the results from the assay of seven different conditions. The differences were minimal (< 2% and most < 1%) indicating that the method is robust.

Conclusion

This application demonstrates an improved IC method for the assay of citrate and phosphate using a Dionex IonPac AS11-HC-4µm microbore column on a Dionex Integrion HPIC system. The improved method has increased sensitivity with LOQs of 0.03 and 0.06 mg/L of phosphate and citrate, respectively, using 25% less sample than the previous method. The method was precise (< 2% RSDs over 4 days) and accurate (95–106% recoveries).

The robustness evaluations showed that the method was affected by variations in temperature and eluent concentration, but baseline separation of citrate and phosphate was maintained. These temperature and eluent variations are unlikely to occur on the Dionex Integrion HPIC system using eluent generation and temperature control in the column and detector compartments. Additionally, the new method doubles sample throughput by reducing the run time to 5 min.

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