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Development and application of a universal method for quantitation of anionic constituents in active pharmaceutical ingredients during early development using suppressed conductivity ion chromatography

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Abstract

A universal method for quantitation of anionic substances in active pharmaceutical ingredients (API) during early development was developed using ion chromatography (IC). The method was developed to allow rapid characterization of APIs in support of early clinical studies The method parameters were chosen to allow quantitation of monovalent, divalent, and trivalent inorganic ions as well as monvalent and divalent carboxylic acids. These parameters were also chosen to ensure appropriate performance for regulated analyses using less than 10 mg of API per replicate. The method was applied to and validated for a range of anionic analytes in APIs of varying hydrophobicity to demonstrate applicability to various analyses encountered during early development of pharmaceuticals.

Keywords: Ion chromatography; Universal method; Active pharmaceutical ingredient; Conductivity detection; Counterion; Method development; Method validation

1. Introduction

In the pharmaceutical industry, there is an increasing trend to conduct small, rapid clinical studies to aid in the selection of active pharmaceutical ingredient (API) candidates for further development. This strategy minimizes time available for development and validation of the analytical methods needed to support these studies. One strategy commonly used to minimize method development and validation time required is the universal method, i.e. a set of experimental conditions used to determine a range of analytes commonly encountered. This work describes the development, validation, and application of a universal method for anions in APIs.

Salts of APIs, either anionic or cationic, are prepared for a number of purposes [1]. Analysis of anions in APIs is also carried out for two reasons. The first is demonstration of the appropriate amount of the anionic counterion in the salt, which is an important step in characterization of the API [2,3]. The other reason is to assess amounts of anionic synthetic impurities and degradation products [4–7].

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In considering methodologies for a universal method for anions typically found in pharmaceuticals, there are a number of techniques which have been previously used for this type of analysis. Examples include potentiometic titrations [3], ion-selective electrodes [8–10], complexometric methods, chromatographic methods with indirect UV detection [11,12], capillary electrophoresis methods with indirect UV detection [3,13–15], chromatographic methods with light-scattering detection [3], and chromatographic methods with suppressed [3,4,6] and non-suppressed conductivity detection [2]. The commercial availability of integrated instrumentation, availability of appropriate chromatographic conditions and columns, and high sensitivity led to the selection of ion exchange chromatography with suppressed-conductivity detection, also known as ion chromatography (IC).

This work describes the development of the chromatographic and sample preparation parameters for a universal anion method by IC. It also demonstrates that the methodology can be validated for use in regulated environments.

2. Experimental

2.1. Ion chromatography

The water used in all sections of this work was prepared by in-house Milli-Q water systems (18 mΩ, Millipore, Bedford, MA, USA). Citric acid (>98%) and 50% NaOH were obtained from EM Science (Gibbstown, NY). Seven anion standard (fluoride, chloride, nitrite, bromide, nitrate, sulfate, and phosphate) was obtained from Dionex (Sunnyvale, CA). Sodium carbonate (reagent grade) was obtained from Mallinkrot (Paris, KY). Maleic acid, sodium chloride, and sodium formate with purity of 99% or greater were obtained from Aldrich. Amiodarone HCl, Amitriptyline HCl, potassium dihydrogen phosphate, and sodium acetate trihydrate with purity of 98% or higher were obtained from Sigma. Sodium oxalate, sodium propionate, and sodium trifluoroacetate with a purity of 99% or greater were obtained from Fluka (Buchs, Switzerland). HPLC-grade acetonitrile was purchased from Burdick and Jackson (Muskegon, MI). Unless otherwise mentioned, all samples were prepared using water as sample diluent. Standards prepared from Dionex seven anion standard were diluted using volumetric glassware and assigned concentrations based on the manufacturer's certificate of analysis. Unless specifically mentioned, all experiments in this work were performed on a Dionex IC system (Sunnyvale, CA) consisting of a model GP50 pump, a model EG40 eluent generator, a model AS50 autosampler, a model ED50 conductivity/amerometry detector with an 4 mm ASRS-Ulta suppressor, and a model LC25 column heater. Water used for mobile phase was degassed via sonication and vacuum for approximately 20 min prior to use. All mobile phases were made by water and the eluent generator module in the ion chromatography system. The system was equilibrated for 2 h and four prime injections were made prior to injection of initial standards to ensure system equilibration. Data was stored and processed using a Turbochrom data system (PE-Nelson, Shelton, CT).

2.2. Capillary electrophoresis

Potassium dihydrogen phosphate (99%) was obtained from Aldrich. The running buffer was the organic acids buffer obtained from Agilent Technologies (Palo Alto, CA). All samples and standard were prepared using water as sample diluent. Phosphate target concentration was 60 μg/ml. The capillary electrophoresis system used (HP3D) was obtained from Agilient Technologies (Palo Alto, Ca). A 64.5 cm × 75 μm i.d. capillary was used with an effective separation distance of 8.5 cm. A 10 min prerun conditioning rinse (organic acid running buffer) and 2 min postrun conditioning rinse was used. The voltage program consisted of a voltage ramp from 0 to 30 kV in 0.3 min was followed by 0.6 min at 30 kV. Sample injection was for 4 s at 50 mbar followed by a running buffer injection for 4 s at 50 mbar. Indirect detection at 350 nm was used.

2.3. Complexometric determination of phosphate

Potassium dihydrogen phosphate, potassium antimonyl tartrate hydrate, ammonium heptamolybdate tetrahydrate, and L-ascorbic acid were obtained from Aldrich. Sulfuric acid was procured from Mallidkrodt (Paris, KY). All samples and standards were prepared using water as sample diluent. Phosphate target concentration was 1 mg/l. Potassium antimonyl tartrate

solution was prepared by weighing approximately 1.38 g into a 500 ml volumetric flask and diluting to volume with water. Ammonium molybdate solution was prepared by weighting 20 g of ammonium heptamolybdate tetrahydrate into a 500 ml volumetric flask and diluting with water. Combined analysis reagent was prepared by first mixing 20 ml of 5N sulfuric acid, 2 ml of potassium antimonyl tartrate solution, and 6 ml of ammonium molybdate solution and letting the solution sit for 30 min. At this point, 0.01 M ascorbic acid was then added; the combined analysis reagent is stable for 4 h. Next, 25 ml of the sample or standard solution was then mixed with 4 ml of combined analysis reagent and diluted to 100 ml with water. The sample and standard solution were allowed to sit for 30 min prior to measurement of visible absorption at 880 nm. All measurements were taken within 60 min of sample preparation. Absorption values were acquired using a model 8453 UV-Vis spectrophotometer from Agilent Technologies (Palo Alto, CA).

2.4. Potentiometric titration of chloride

HPLC-grade methanol was obtained from Burdick and Jackson (Muskegon, MI). Sodium chloride (99.999%) was obtained from Aldrich.

Concentrated nitric acid was procured from EM Science (Gibbstown, NY) Samples and standards were prepared by diluting appropriate amounts of sample or sodium chloride standard into 10 ml of methanol and 50 ml of 2% nitric acid. The samples were then titrated using 0.05N silver nitrate.

The model DL70ES titrator used was from Mettler (Columbus, OH).

2.5. Acid–base titration of acetate

Potassium bipthalate (99%) and NaOH pellets were obtained from Aldrich. HCl concentrate was obtained from Anachemia (Rouses Point, NY). Samples and standard were prepared by diluting appropriate amounts of sample or potassium bipthalate into 50 ml of water. Acetate was liberated from the compound A salt by addition of 6 ml of 0.1N HCl. The samples and standards were titrated using 0.1N NaOH. The model DL70ES titrator used was from Mettler (Columbus, OH).

2.6. Validation of IC method

Linearity was evaluated by dilution of a stock solution from approximately 2–500% of the analysis concentration for inorganic anions. The linearity range evaluated for acetate was 80–120% of the analysis concentration. Accuracy and precision were assessed by calculating the average and relative standard deviation of triplicate API samples. Accuracy was assessed as percent recovery versus a reference method if available or theoretical weight percent if a reference method was not available. Specificity was determined by comparison of the accuracy samples to the diluent blank. Stability of accuracy samples and standards were evaluated at approximately 2, 4, and 9 days or only at 6 days.

Sample preparation procedures were dependent upon the nature of the API. For highly-soluble APIs, sample preparation consisted of dissolving 5–7 mg of the API in an appropriate volume of water prior to injection. Samples sizes were selected to be the smallest possible which could still be accurately weighed according to the United States Pharmacopoeia on available laboratory balances. For poorly-soluble APIs, sample preparation was compound-specific. For both model compounds, API sample weight was approximately 6 mg. Amiodarone was first was dissolved in 35% acetonitrile. It was then precipitated with an amount of 50 mM NaOH designed to bring the final solution concentration to 2 mM NaOH after dilution to volume with water. For Amitriptyline, two different procedures were used. In experiments using 35% acetonitrile in the mobile phase, Amitriptyline was dissolved in 35% acetonitrile in water. For experiments without acetonitrile in the mobile phase, Amitriptyline was prepared in the same manner as Amiodarone. Samples with precipitated API were filtered through a 0.45 μm PTFE syringe filter prior to injection.

3. Results and discussion

3.1. Development of ion chromatographic conditions

The methodology was developed to separate counterions typically used in APIs ranging from singly charged anions such as acetic acid and chloride to

Table 1
Method conditions

Parameter	Initial conditions		Final conditions	
Injection volume (μl)	25		25	
Column	IonPac AS11 (250 mm × 4 mm) with AG11 guard column		IonPac AS11HC (250 mm × 4 mm) with AG11HC guard column	
Column temperature (°C)	35		35	
Flow rate (ml/min)	2		1	
Suppressor	ASRS Ultra, 4 mm		ASRS Ultra, 4 mm	
Suppressor mode	Recycle		External water	
Suppressor current (mA)	300		100	
Detection	Conductivity, suppressed		Conductivity, suppressed	
Gradient	Time	mM KOH	Time	mM KOH
	0	0.5	0	3
	2.5	0.5	5	3
	6	5	12	5
	18	38	36	35
	18.2	0.5	36.2	3
	25	0.5	50	3

triply-charged anions such as phosphate and citrate. Initial chromatographic conditions were selected based on conditions from the manufacturer's literature [16], which are shown in Table 1.

These conditions were modified to minimize the amount and impact of random peaks observed in the chromatograms, which were attributed to the high suppressor current used. The suppressor regeneration mode was changed from recycle mode to external water mode. Flow rate was reduced twofold and gradient times were increased twofold to minimize suppression requirements of the system while maintaining selectivity. In addition, the column was changed from a Dionex AS11 to a Dionex AS11HC. This served to increase the starting ionic strength of the gradient which in turn minimized the differential between the suppression capacity of the suppressor and the suppression requirements of the mobile phase. Final chromatographic conditions are also listed in Table 1, and the resulting chromatogram of 14 relevant anions is shown in Fig. 1.

Analysis concentrations were selected by performing linearity studies and selecting the concentration which resulted in less that 5% interference from the largest stray peak in the run. The concentrations determined were 4 mg/l of fluoride; 6 mg/l of chloride, 20 mg/l of nitrite, 20 mg/l of bromide, 20 mg/l of nitrate, 30 mg/l of sulfate, and 30 mg/l of phosphate. Because this method was intended to determine carboxylic acids as well as inorganic anions, a similar analysis was performed using acetic acid and trifluoroacetic acid. The concentration determined for both analytes was 20 mg/l. It should be noted that the acetic acid responses were clearly non-linear over the range of concentration studied. This is due to the fact that acetate is not completely ionized. For an excellent discussion of this phenomenon and other difficulties with non-linear response in carboxylic acids, see [17].

An additional consideration in the determination of method conditions is the aqueous solubility of the API. In many cases, APIs may be poorly soluble in the highly basic aqueous mobile phases used for anion determination in IC. This could lead to precipitation of the API in the chromatographic system and the potential for column fouling and excessive back pressure. This problem was addressed in two ways. The first was to include 35% (v/v) acetonitrile in the mobile phase and sample diluent to support the solubility of the API. The acetonitrile concentration was fixed to eliminate the need for method development. The choice of acetonitrile over methanol was arbitrary. Higher concentrations were not used to avoid the use of chemical regeneration of the suppressor. The second was to modify sample preparation to include a step to precipitate and filter the API after initial

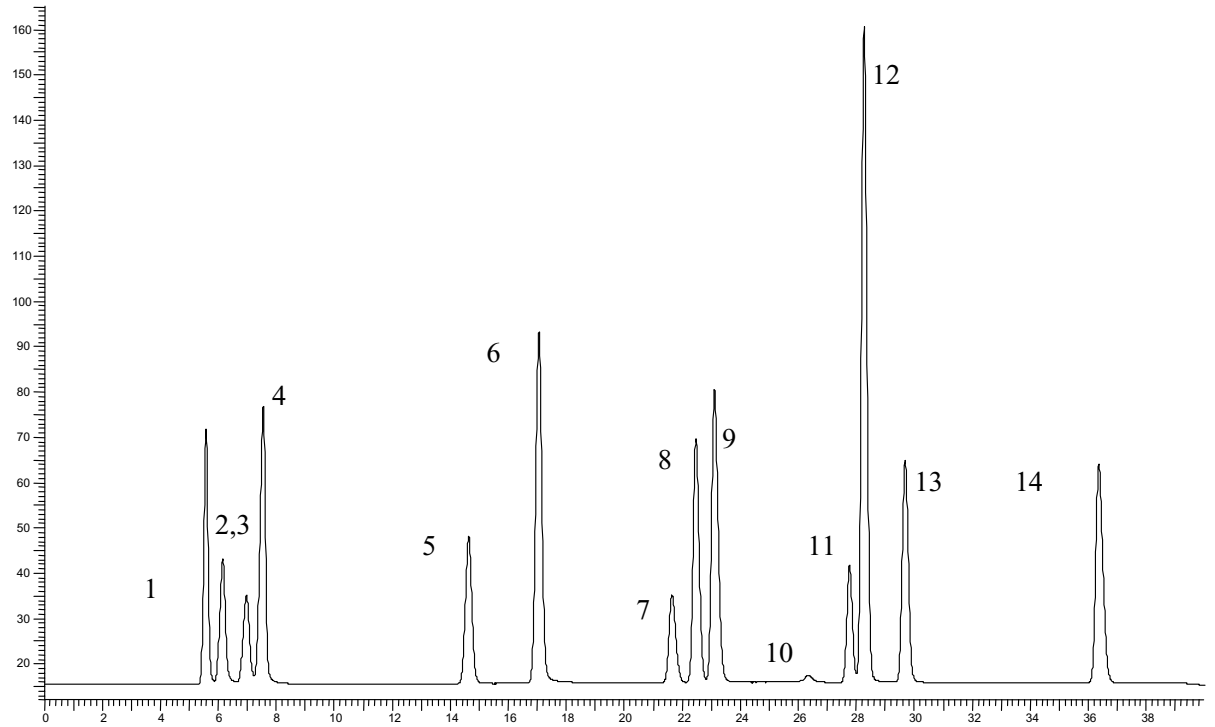


Fig. 1. Chromatogram of a number of anions of interest. The peaks, starting from 1, are fluoride, acetate, propionate, formate, chloride, nitrite, trifluoroacetate, bromide, nitrate, carbonate (trace in blank), maleate, sulfate, oxalate, and phosphate. The *y*-axis is response in mV, and *x*-axis is retention time in minutes.

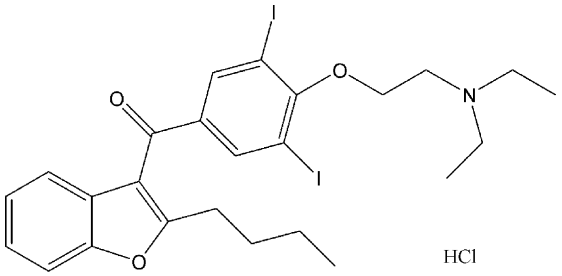
dissolution. Selection of the appropriate method for sample preparation was performed as follows. Solubility of the model APIs (Fig. 2) at the analysis concentration was evaluated in water, 35% (v/v) acetonitrile, 3.75 mM NaOH, 3.75 mM NaOH with 35% (v/v) acetonitrile, 37.5 mM NaOH, and 37.5 mM NaOH with 35% (v/v) acetonitrile.

These solutions were chosen as models for the initial and final mobile phase conditions with and without the acetonitrile mobile phase modifier. The exact concentrations were chosen for facile dilution from 50% (w/w) solutions of NaOH. The results of these solubility tests were used to determine sample preparation procedures. Amiodarone was soluble only in the 35% (v/v) acetonitrile. Therefore, it was dissolved in 35% (v/v) acetonitrile, and the free base was precipitated with NaOH. The solution was then filtered prior to injection. Amitriptyline was soluble in water and in all diluents containing 35% (v/v) acetonitrile. So for experiments using 35% (v/v) acetonitrile in the mobile phase, it was dissolved in 35% (v/v) acetonitrile. For experiments without 35% (v/v) acetonitrile in the mobile phase, it was also dissolved in 35% (v/v) acetonitrile, precipitated with NaOH, and filtered prior to injection.

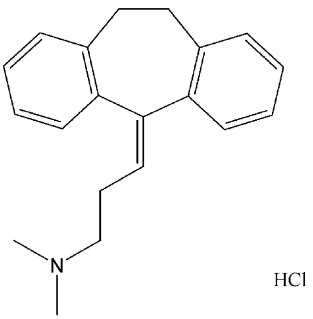
3.2. Validation of the method for highly-soluble API

The method was applied to the determination of acetate, chloride, and phosphate counterions in three different water-soluble APIs to demonstrate its applicability to a variety of analytes. Compound A was a proprietary trihydrochloride salt; compounds B and C are shown in Fig. 3.

Validations were undertaken assuming an accuracy values of 90–110% were appropriate; this value was arrived at in consultation with individuals responsible for API solid-state determination. Other validation parameters are given in Table 2; these parameters are

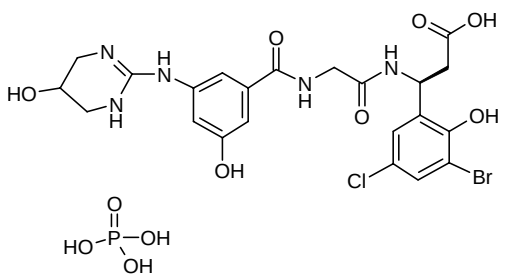


Amiodarone HCl

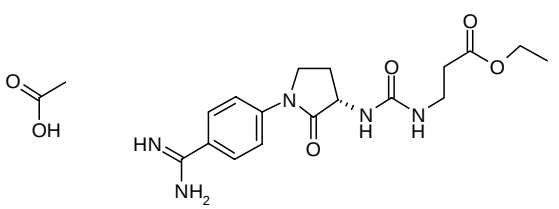


Amitriptyline HCl

Fig. 2. Structures of water-insoluble model APIs.



Compound B



Compound C

Fig. 3. Structures of water-soluble APIs.

Table 2
Validation parameters

Parameter	Target value
Linearity	Visual inspection of a plot of signals as a function of analyte concentration should show a linear relationship. Deviations from single-point fit must meet accuracy requirements at all points
Accuracy (%)	90–110%
Precision (%)	Repeatability: R.S.D. <3
Specificity (%)	No interference >2
Stability (%)	Recovery >95% from initial values
System suitability (%)	R.S.D. of analyte peak in standard less than 2.0

used for the validations of the method for all compounds in this work.

The linear ranges were approximately two orders of magnitude for inorganic anions (Table 3).

Linearity was evaluated for all of the anions in the commercially-available anion mixture used to prepare the linearity samples. The linearity range was determined comparing the percent difference (% bias) between the raw peak areas and the theoretical peak areas determined from a single-point line based on the analysis concentration forced through zero. The single-point concentration in Table 3 is level III. While the validation criterion was to have this percent bias less than 10%, the linear ranges were conservatively estimated using only points with a bias less than 5%. For acetate, a much smaller linear range was found as expected (Table 4).

Even at 80–120% of the analysis concentration, the biases between the actual peak areas versus the single-point fit show the non-linear nature of the response. As Table 4 showns, a least-squares fit of all the data points reduces the bias between the calibration line and the actual results considerably. In response to this, quantitation of all acetate samples was performed versus a least-squares fit of standards at 80, 100, and 120% of the analysis concentration.

Accuracy and precision results for the highly-soluble APIs were excellent. For compound A, accuracy was 102.7% and precision was 2.0% for the chloride counterion. For compound B, accuracy was 96.1% and precision was 0.3% for the phosphate counterion. And for compound C, accuracy was 99.9% and precision was 0.5% for the acetate counterion.

Table 3
Linearity results for inorganic anions

Level	Concentration (mg/l)						
	Fluoride	Chloride	Nitrite	Bromide	Nitrate	Sulfate	Phosphate
I	20	30	100	100	100	150	150
II	10	15	50	50	50	75	75
III	4	6	20	20	20	30	30
IV	2	3	10	10	10	15	15
V	0.8	1.2	4	4	4	6	6
VI	0.4	0.6	2	2	2	3	3
VII	0.2	0.3	1	1	1	1.5	1.5
VII	0.08	0.12	0.4	0.4	0.4	0.6	0.6
Linear range	0.2–10	0.3–30	0.4–20	4–100	4–100	6–150	1.5–150

Specificity and system suitability were excellent for all three anions. No blank response was observed for acetate, chloride, and phosphate. The system suitability criterion, relative standard deviation the peak area of six injections of the standard, was less than 1% in all runs which exceeded the requirement of 2%.

For sample and standard stability, the acetate samples and standards showed differences from the chloride and phosphate samples and standards. The chloride and phosphate samples and standards were stable for 9 days of benchtop storage. The acetate samples were stable only for 2 days on the benchtop but for 9 days when refrigerated.

3.3. Validation of the method for poorly-soluble APIs

The method was applied to the determination of chloride in Amitriptyline and Amiodarone using mobile phases with and without 35% (v/v) acetonitrile in the mobile phase. For the experiments with mobile phase containing 35% (v/v) acetonitrile, analysis concentration was 15 mg/l versus 6 mg/l without the acetonitrile.

Table 4
Linearity results for acetate

Nominal concentration (mg/l acetate)	Bias from single-point fit (%)	Bias from full fit (%)
24	5.2	−0.3
27	2.9	0.5
30	0	−0.2
33	−1.8	−0.04
36	−3.3	0.03
Slope (μV s l/mg)	22113	17519
Intercept	0	138748

Selectivity and baseline noise were changed by the addition of 35% (v/v) acetonitrile to the mobile phase. There was less retention with the organic modifier in the mobile phase, and several peaks switched elution order (Fig. 4).

Nitrite and nitrate were particularly susceptible to this effect. Nitrite coeluted with chloride under these conditions. The baseline noise was also impacted by the change (Fig. 5). These changes had small but noticeable effects on the validation results.

For linearity, the 35% (v/v) acetonitrile had a linear range from 3 to 30 mg/l chloride versus 0.3–30 without 35% (v/v) acetonitrile. The loss of linearity at the low end of the range is attributed to the additional baseline noise. There is also some loss of sensitivity. The slope of the calibration line is approximately 57,000 μV s l/mg for the 35% (v/v) acetonitrile mobile phase and approximately 85,000 μV s l/mg without actonitrile in the mobile phase.

Accuracy was unchanged by addition of the acetonitrile while precision was impacted (Table 5).

The considerable increase in baseline noise for the acetonitrile-bearing mobile phase directly affects the precision results. System suitability results were also somewhat affected. The average standard peak area R.S.D. was 1.0 without acetonitrile and 1.3% with acetonitrile. Specificity was not significantly impaired by the actonitrile in the mobile phase.

3.4. Sample size requirements

The previous experiments in this work have shown how characterization of the counterions in APIs can be carried out with regulatory rigor on minimal amounts

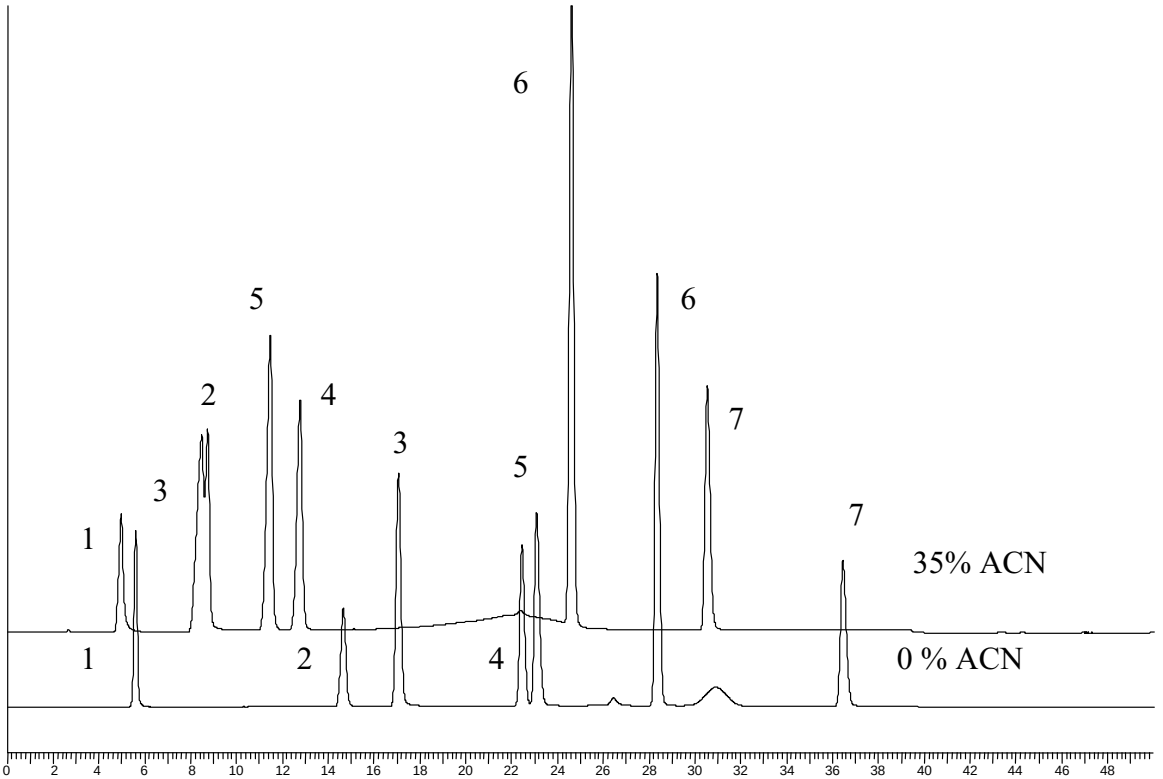


Fig. 4. Selectivity differences between 0 and 35% acetonitrile in the mobile phase. Peak 1 is fluoride, peak 2 is chloride, peak 3 is nitrite, peak 4 is bromide, peak 5 is nitrate, peak 6 is sulfate, and peak 7 is phosphate. The *y*-axis is response in mV, and *x*-axis is retention time in minutes.

Table 5
Accuracy and precision results for poorly-soluble APIs

Mobile phase	Amitriptyline accuracy	Amitriptyline precision	Amiodarone accuracy	Amiodarone precision
With 35% (v/v) Acetonitrile	99.9	1.4	100.6	1.1
Without 35% (v/v) Acetonitrile	99.9	0.2	100.4	0.2

of material. Another commonly encountered challenge is performing the analysis on amounts of material which cannot be weighed accurately by regulatory definitions. To test whether or not this type of analysis was appropriate, the sample preparations were scaled down by a factor of five to use approximately 1 mg of sample. Triplicate preparations of 1 mg sample weighings were made on the same type of balance (1 μg readability, ±4 μg linearity). One lot of all five compounds was tested (Table 6). The reduction in sample size leads to no practical difference in the results.

Table 6
Comparison of ∼5 mg versus ∼1 mg sample size results

Compound	Sample wt. (mg)	Recovery (%)	R.S.D. (%)
Amiodarone	5, aqueous	100.4	0.2
Amiodarone	1, aqueous	102.5	0.6
Amitriptyline	5, aqueous	99.9	0.2
Amitriptyline	1, aqueous	102.6	1.4
Compound A	5	102.7	2.0
Compound A	1	96.8	1.4
Compound B	5	96.1	0.3
Compound B	1	93.7	0.6
Compound C	5	99.5	0.5
Compound C	1	99.8	0.3

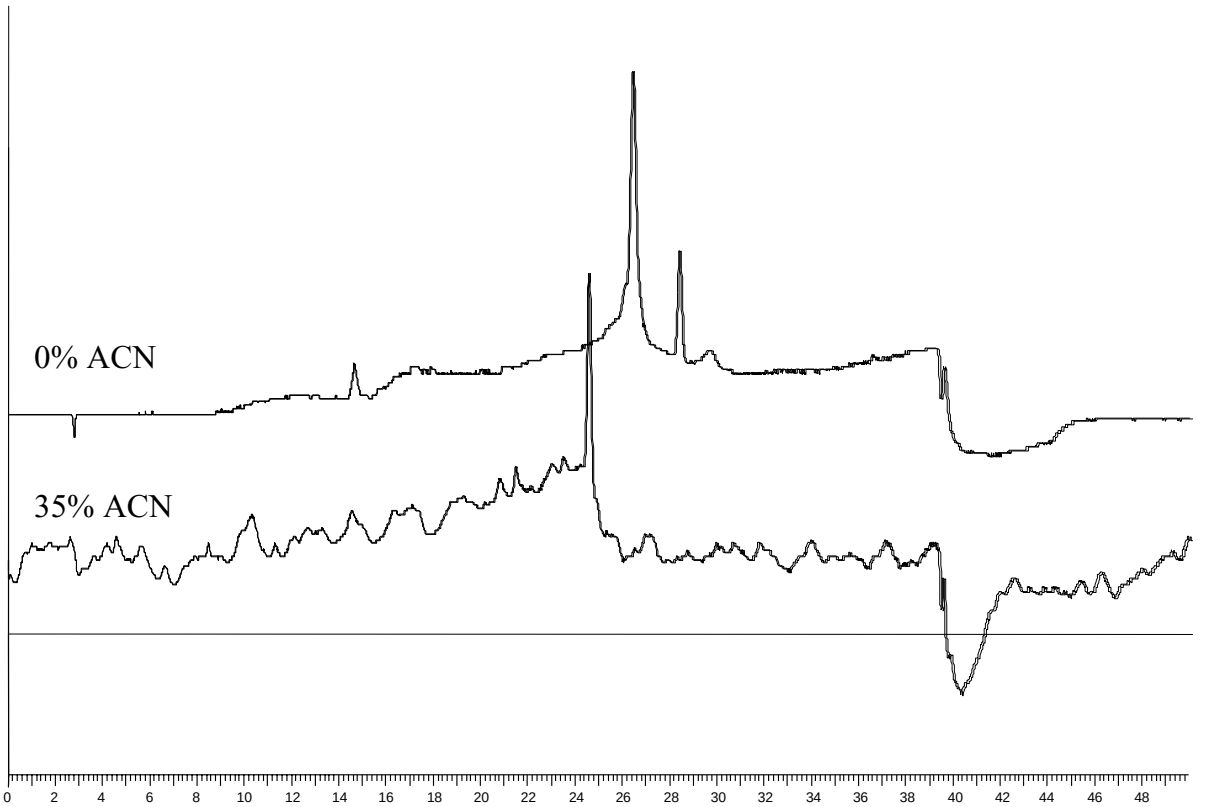


Fig. 5. Comparison of baseline noise between 0% acetonitrile and 35% acetonitrile in the mobile phase. The *y*-axis is response in mV, and *x*-axis is retention time in minutes.

Table 7
Comparison of results from universal IC anion method to other methodologies and theoretical values

Compound (analyte)	Lot no.	Original methodology	Original (wt.%)	IC (wt.%)	Theoretical (wt.%)	Corrected theoretical (wt.%)[a]	Mole ratio original/IC
A (chloride)	1	Titration	19.5	20.0	19.0	17.8	3.06/3.12
A (chloride)	2	Titration	17.3	18.0	19.0	18.4	2.82/2.94
B (phosphate)	1	Complexometric	12.2	11.7	13.4	12.6	0.97/0.93
B (phosphate)	2	Complexometric	12.9	11.6	13.4	12.6	1.02/0.92
B (phosphate)	3	Complexometric	12.7	12.1	13.4	12.5	1.02/0.97
B (phosphate)	1	Capillary electrophoresis	12.8	11.7	13.4	12.6	1.02/0.93
B (phosphate)	2	Capillary electrophoresis	11.9	11.6	13.4	12.6	0.94/0.92
B (phosphate)	3	Capillary electrophoresis	13.0	12.1	13.4	12.5	1.04/0.97
C (acetate)	1	Titration	14.1	14.1	14.25	–	0.99/0.99
C (acetate)	2	Titration	14.1	14.0	14.25	–	0.99/0.98
C (acetate)	3	Titration	14.0	13.9	14.25	–	0.98/0.98
C (acetate)	4	Titration	14.2	14.0	14.25	–	1.0/0.98

[a] Corrected for water and solvent content.

3.5. Comparison of IC results to reference methods

During the course of these experiments, the counterion content of additional lots of each compound were determined to compare the relative accuracy of the IC conditions to methodologies used previously (Table 7). There were no practical differences in the results. The other techniques consume approximately 75–100 mg per replicate, while the IC method uses 5–7 mg per replicate.

4. Conclusions

Chromatographic and sample preparation conditions were developed and validated for a universal anion analysis method applicable to analyses required in early pharmaceutical development. The method can perform regulated analyses on minimal amounts (5–7 mg) of material. The method was demonstrated to be validatable for a range of anions from acetic acid to phosphate with appropriate accuracy, precision, and linearity. It was also shown to be amenable to hydrophobic APIs with appropriate sample preparation techniques. It can also be applied with reasonable accuracy and precision to samples as small as 1 mg of API. And finally, the IC method was shown to give results comparable to reference methods while using considerably less material.

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