Evaluation of Methods for the Simultaneous Analysis of Cations and Anions Using HPLC with Charged Aerosol Detection and a Zwitterionic Stationary Phase

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Abstract

This paper describes the development and qualification of a method capable of analyzing inorganic ions as salts and counter-ions of both active pharmaceutical ingredients and other compounds such as lysine. The use of a polymeric zwitterionic column with a binary high-performance liquid chromatography gradient enables the separation of several anions and cations in a single run. A generic gradient (method #1) was developed and validated with respect to specificity, correlation, intermediate precision, accuracy, and sensitivity (limits of quantitation and detection) for four anions and two cations. Furthermore, the ability to alter chromatographic selectivity by simple gradient manipulation (without altering the mobile phase composition or column type) is demonstrated for nine anions and three cations (method #2). The simultaneous measurement of cations and anions at the parts per billion level using the Corona charged aerosol detector with zwitterionic chromatography–polymeric hydrophilic interaction chromatography is a viable alternative to traditional techniques used for ion analysis.

Introduction

The need to analyze inorganic cations and anions stretches across many fields, ranging from pharmaceutical formulations and product characterization to environmental analysis. The use of ion chromatography (IC) with a conductivity detector for the analysis of inorganic anions is the most common technique employed today. The analysis of cations uses a variety of different techniques including IC-conductivity detection, atomic adsorption spectroscopy, and inductively coupled plasma with atomic emission spectroscopy or mass spectroscopy (MS) (1). These techniques have proven themselves to be capable of the analysis; however, they are highly specific and, therefore, can become costly. The basic IC system consists of an autosampler and pump along with an ion exchange column, an ion-suppressor, and a conductivity detector (2). Even though suppressor technology has improved, these techniques by their design do not allow for the simultaneous analysis of anions and cations in a single run. Due to time requirements in changing an IC system from one ion to another, many laboratories accept the expense of having dedicated, platform-dependent instruments for each suite of analytes.

The pharmaceutical industry represents one of the fields that requires both sensitive and reproducible methods for the analysis of counter-ions that are an integral part of the active pharmaceutical ingredient (API). The use of various inorganic or organic counter-ions is now an important part of the drug development process. According to the data from the Cambridge Structural Database, six of the top ten pharmaceutically acceptable counter-ions occur as salts of inorganic anions. The salt formation is used to selectively alter physicochemical characteristics of the drug, such as solubility, stability, and hygroscopicity (3). The traditional methodology for testing these compounds uses IC with conductivity detection techniques but, as discussed previously, this can be somewhat limited as only anions or cations are measured in a single run. An alternative approach uses strong anion or cation exchange columns with evaporative light scattering detection (ELSD), but this means of detection generally lacks sensitivity and precision (see the following).

Electrostatic ion chromatography was first introduced by Wenzhi Hu and colleagues in 1993 (4). This technique was further refined and was later named zwitterionic chromatography (ZIC). There are primary and secondary interactions described by the column manufacturer SeQuant when using the ZIC–hydrophilic interaction chromatography (HILIC) column. The primary interactions are those typical of HILIC, resulting from the hydrophilic interactions and the partitioning of the solvent in the column with a water-enriched liquid layer. This then allows for separation by partitioning solutes into this hydrophilic environment. A recent publication evaluating the differences between various HILIC columns and the ZIC–HILIC employed in this study for small polar compounds found that there were many similarities amongst these columns (5). Other work on the evaluation of ion analysis has also indicated that secondary electrostatic interactions become more prevalent with the stationary phase on the ZIC–HILIC

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(6). The ZIC–polymeric (p) HILIC column contains negatively charged sulfonate groups and positively charged quaternary amine groups. The proximity of these groups results in the zwitterionic mechanism, which allows for the retention of both cations and anions in a single run.

Recently, the work of Risely and Pack using a silica-based ZIC–HILIC column and ELSD elegantly illustrated the ability of this approach to simultaneously measure positive and negative counter-ions of pharmaceuticals (6). They also reported how manipulation of the mobile phase pH and buffer strength could be used to alter analyte retention and resolution. Unfortunately, analyte detection by ELSD suffers from a number of significant limitations including poor precision, average sensitivity, narrow dynamic range, wide inter-analyte response, and issues related to the nature of standardization/calibration curves (7,8). Many of these limitations can be overcome by using charged aerosol detection (9,10). For example, Liu et al. recently published a validated method for the measurement of etidronate disodium and its phosphate and phosphite impurities using a mixed-mode column and charged aerosol detection (11).

The principle of Corona charged aerosol detection (CAD) differs from other universal detection methods. The eluent from the high-performance liquid chromatography (HPLC) column enters the instrument where it is nebulized with pressurized gas, which is typically nitrogen or air. The residual mobile phase is evaporated from the aerosol as they travel down the drying tube, leaving solid analyte particles. At the same time, a second stream of gas passes a corona discharge needle where the gas becomes positively charged. The two gas streams collide, resulting in charge being transferred to the particles. After high mobility species are removed at an ion trap, the remaining particles pass to a collector where the charge is measured with a very sensitive electrometer. Because the entire process involves particles and direct measurement of their charge, the technique is highly sensitive, consistent, and has a broad dynamic range. The measurement of particle charge provides a more universal response independent of the chemical properties of the analyte. The sensitivity of detection generally falls in the low nanogram level for any non-volatile compound.

The Risley and Pack paper was used as the theoretical basis for the development of the methods reported here. Cations and anions were simultaneously resolved on a polymeric zwitterionic column using gradient elution, and were measured using a CAD. This analytical method was validated with respect to range, linearity, specificity, sensitivity, accuracy, and precision. The manipulation of the gradient and its effects on analyte resolution is also discussed.

Experimental

Reagents and standards

Ammonium acetate (≥ 99.0%, Fluka BioChemika #09689) and acetic acid (LC–MS-grade, Fluka #49199) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (ACN) (Omnisolve High Purity Solvent EMD #AX0142-1) and methanol (J.T. Baker CMOS Grade, #90703-05) were obtained from VWR (West Chester, PA). Isopropanol (electronic grade #64028) was purchased from Doe & Ingalls (Medford, MA). Water (deionized, 18.2 Mohm-cm) was prepared in-house with a PureLab Ultra ELGA Labwater system from US Filter (Lowell, MA). Ammonium nitrate (Sigma-Aldrich #256064), ammonium chloride (Sigma-Aldrich #254134), ammonium sulfate (Sigma-Aldrich #204501), sodium acetate (Sigma-Aldrich #229873), potassium acetate (Sigma-Aldrich #25578), quinine sulfate dihydrate (Fluka #22640), diclofenac sodium salt (Sigma #D-174), acesulfame K (Fluka #04054), and lysine dihydrochloride (Sigma-Aldrich #62910) were purchased from Sigma-Aldrich.

Equipment

The repeatability and standardization work was completed on an Agilent 1200 Series HPLC system with a CAD (model # 70-6350) from ESA Biosciences, Inc. (Chelmsford, MA). The Agilent system consisted of a degasser (Model #G13798), a binary SL Pump (model #G13128B), and an HP-ALS SL autosampler (model # G1367C) (Prospect Heights, IL). A SeQuant ZIC(R)-pHILIC (5 µm, 4.6 × 150 mm) column was used (Nest Group Inc., Southborough, MA). Confirmation and API data were generated on a Shimadzu Prominence HPLC System consisting of a DGU-20A5 degasser, two LC-20AD pumps, a SIL-20AC autosampler, and a CBM-20A communications bus. The mobile phase pH was measured using a Mettler-Toledo MP225 pH meter (Columbus, OH) standardized with pH 4 and 7 buffers. Agilent 1-mL polypropylene vials (5182-0567) and caps (5181-1512) were used throughout the study to minimize potential interferences from sodium and borosilicate ions found in glass HPLC vials.

Chromatographic conditions

Ammonium acetate buffer solution, with pH adjusted to 4.7 using acetic acid, was used in the preparation of both mobile phases A and B. For mobile phase A, the final composition after dilution with organic components was 100 mM ammonium acetate–ACN–isopropanol–methanol (15:65:20:5). For mobile phase B, the buffer was diluted with HPLC grade water and then organic components were added for a composition of 30 mM ammonium acetate (pH 4.7)–ACN–isopropanol–methanol (50:25:20:5). The autosampler wash solution for the Shimadzu system was ACN–HPLC grade water (80:20). The gradient profile used for method #1 during all work related to validation of the method was as follows: T = 0 (min) 45%B, T = 15 65%B, T = 20 65%B, T = 25 40%B, T = 26 45%B, T = 30 45%B. The gradient profile used with method #2, which illustrates selectivity changes and API work, was as follows: T = 0 20%B, T = 3 20%B, T = 24 70% B, T = 26 70%B, T = 32 15%B, T = 34 20%B, T = 40 20%B. Column temperature was maintained at 30ºC. The CAD was set to the default parameters with a range of 100 pA, the filter was off, and nitrogen gas pressure was at 35 psi.

Standards and sample preparation

Solutions of ammonium nitrate, ammonium chloride, ammonium phosphate monobasic, ammonium sulfate, sodium acetate, and potassium acetate were prepared at 10 mg/mL in deionized water. One milliliter of each of the standard solutions was combined together and then diluted to 10 mL. A 2 mL volume of this solution was then transferred to 8 mL of ACN. A serial dilution was then used to prepare standard concentrations

of 100, 50, 25, 12.5, 6.3, 3.2, 1.6, and 0.8 µg/mL in ACN–water (80:20). An aliquot of each of the solutions was then transferred to the polypropylene HPLC vials. These standards were labeled as standards 1 (100 µg/mL) through 8 (0.8 µg/mL). Some additional ions, not part of the validation study, were prepared as single points, at approximately 30 µg/mL in ACN–water (80:20). Accuracy samples were weighed and then diluted to their final concentration in ACN–water (80:20). The 0.1% spike solutions were prepared by adding equal volumes of a diluted impurity solution and an API sample solution to obtain an impurity concentration of 0.1% (w/w) relative to the API.

Method development

The initial goals were to develop a method which would achieve suitable resolution between sodium, potassium, chloride, sulfate, and phosphate. The early work with the ZIC–pHILIC column using isocratic conditions with an ACN aqueous buffer mobile phase resulted in decreased column efficiency over time. Running gradient conditions from low to high aqueous at 1 mL/min, similar to those in the Risely paper, resulted in higher column backpressures than those recommended by the manufacturer of the polymer column. It was determined that optimal column performance was obtained between 0.5 and 0.75 mL per min. At these flow-rates, the addition of organic modifiers is needed to maintain peak shape and elute all ions within a reasonable time period. The addition of methanol was found to have a large effect on the elution of the divalent anions. Isopropanol had a similar but less dramatic effect. Several concentrations of buffer, between 25 and 150 mM, were evaluated to determine the optimal point for separation of the ions of interest. The effect of different ammonium acetate buffer concentrations on peak retention was found to correlate well with the results reported by Risely and Pack using a silica-based ZIC–HILIC column. To minimize baseline drift due to gradient conditions, the total mass of ammonium acetate in mobile phases A and B were kept equal while the aqueous concentration changed. The final conditions listed in gradient method #1 were determined to provide the optimal resolution for all of the ions of interest in the shortest time period.

Results and Discussion

Validation parameters

The acceptance criteria, which were defined prior to these experiments, indicated that the results should be within ± 5% of target values for accuracy and recovery when analyzed using gradient method #1 (12). For precision data, the accepted % relative standard deviation (RSD) value for an $N = 4$ was set at ≤ 5%. The robustness of the method was not completely tested according to the guidelines presented in FDA Q7A. Full validation with consideration of robustness includes stability investigations, changes to mobile phase pH, buffer concentrations, and organic composition, as well as different column lots and manufacturers, flow-rate changes, temperature changes, and analyst-to-analyst variations. Although some of these parameters are discussed in the present work, this type of analysis is important for the end use of the system where a more thorough examination is required for use in a current good manufacturing practice environment. The work presented in this paper was done for feasibility purposes and therefore this level of testing was not warranted.

Range and linearity

The injection volume was 10 µL for solutions of approximately 800 ppb to 100 ppm of each of the ion salts. The peak areas for three injections of each of the eight standard solutions of ions were plotted versus the mass on column of the corresponding salts. Standardization curves for the resulting data were then fitted to both linear and 2nd order

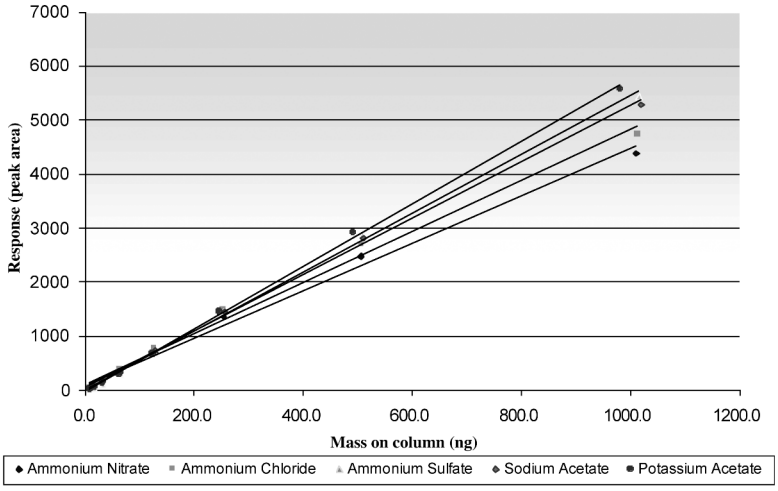

Figure 1. Calibration curves (average of three injections each for 8 levels ~1 µg to ~ 8 ng on column) for the ammonium or acetate salts of the ions, analyzed using a linear regression.

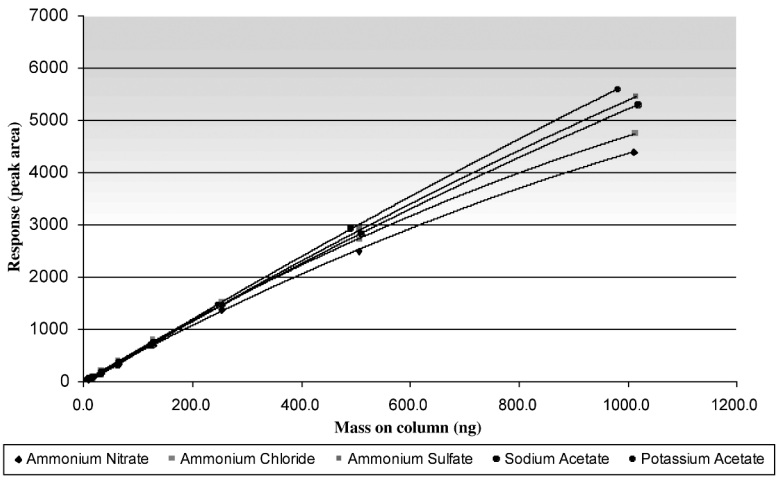

Figure 2. Calibration curves (average of three injections each for 8 levels ~1 µg to ~8 ng on column) for the ammonium or acetate salts of the ions, analyzed using a 2nd order polynomial.

polynomials, as shown in Figures 1 and 2, respectively. The dynamic range of the detector was demonstrated to span from 8–1000 ng on column. Over this range, the linear curves for each of the ions demonstrated a slight decrease in response at the higher concentrations, and this calibration method did not significantly influence the acceptance of accuracy criteria for this assay. The slight decrease in detector signal at higher concentrations had minor effects on the linear regression of the data, which had correlation coefficients (R^2) greater than or equal to 0.993; whereas the correlation coefficients for the 2nd order polynomial fits showed some improvement over the linear fits and produced an R^2 value greater than 0.999 for each of the ions. All correlation coefficients are presented in Table I.

Table I. Correlation Results for Full Range and Target Curves

	8-point curves		3-point curve
Analyte	Polynomial	Linear	Linear
Nitrate	0.9998	0.9937	0.9995
Chloride	0.9998	0.9931	0.9989
Phosphate	0.9991	0.9984	0.9986
Sulfate	0.9999	0.9983	0.9972
Sodium	1.0000	0.9985	0.9994
Potassium	0.9999	0.9991	0.9980

Table II. Inter- and Intra-Day Reproducibility of Retention Times and Peak Areas

	Day 1 (n = 5) % RSD		Day 2 (n = 5) % RSD		Day 4 (n = 6) % RSD		Day 7 (n = 6) % RSD		All points % RSD	
Analyte	Area	R.T.	Area	R.T.	Area	R.T.	Area	R.T.	Area	R.T
Nitrate	1.2	0.04	1.4	0.16	1.0	0.02	0.9	0.02	2.7	0.83
Chloride	0.5	0.05	1.3	0.16	1.2	0.02	0.6	0.02	2.6	1.3
Phosphate	4.2	0.05	2.4	0.22	3.1	0.05	3.4	0.03	5.3	2.2
Sulfate	1.7	0.06	0.8	0.25	2.2	0.07	1.3	0.08	4.7	2.7
Sodium	1.9	0.02	1.3	0.05	1.7	0.03	1.5	0.09	3.3	1.0
Potassium	1.2	0.02	1.9	0.13	1.2	0.05	1.4	0.15	3.5	0.99

Table III. Accuracy Results

Sample	Injection concentration µg/mL	Calculated ion	Percent recovery
Sodium phosphate monohydrate	46	Na^+	96.5
		PO_4^{3-}	96.3
Quinine sulfate dihydrate	227	SO_4^{2-}	101.4
Diclofenac sodium salt	120	Na^+	99.8
Acesulfame K	75	K^+	99.0
Lysine dihyrochloride	46	Cl^-	103.6
Ammonium nitrate	15	NO_3^-	103.8

Repeatability and intermediate precision

A standard concentration of 12.5 ppm was used to test repeatability and intermediate precision. The sample contained 12.5 ppm of each of the six different ion salts, and this represents 125 ng on-column of each salt. An experiment conducted over seven days with four time points was performed to test repeatability and intermediate precision. The raw peak areas were analyzed for a total of 22 unique injections over the seven-day study. The inter-day and intra-day results for both peak areas and retention times are listed in Table II. The data for the phosphate ion showed the greatest variation in peak area, and this was attributed to the broad peak shape for this analyte. All values for area variability were less than or equal to 5% RSD. Retention time variability was less than 0.25% RSD for all the same day results. The preparation of a new lot of mobile phase for Day 7 resulted in a slight shift in retention times on the final day of the study.

Accuracy

The accuracy of this method was tested both over the full range, as discussed in the "Range and linearity" section, and with three concentrations, including the target concentration of 12.5 ppm and ± 20% of target. A standard solution, using potassium phosphate monobasic, was employed for this part of the procedure. The calculated concentrations used the average of three points for each sample run interlaced with standards. The linear fit technique for establishing molar concentration versus peak area for each of the salts used for all measurements. The calculated molar amounts of counter-ion were then used to calculate an estimated percent recovery for each ion, as shown in Table III. The experimental values were all within ± 5% of the theoretical values. The accuracy of the method was found to be acceptable (11).

Sensitivity

The sensitivity of the method was established using various injection volumes (10, 20, and 50 µL). The test was completed by preparing a five-point dilution curve at and below the expected limit of detection (LOD). The solutions ranged from 125 ppb to 3.5 ppm for each of the ion salts. These solutions were injected three times each and the signal-to-noise (S/N) and %RSD for the three injections were examined. The limit of quantification (LOQ) was defined by ICH guidelines as a S/N of 10 with acceptable reproducibility with %RSD ≤ 10. The LOD was defined as S/N of 3. The LOQ for the method, using 10 µL injections, was determined to be approximately 1 ppm or 10 ng on column (o.c.) for the phosphate, sulfate, sodium, and potassium salts. The LOQ value for the nitrate and chloride salts was determined to be between 250 and 500 ppb (2.5 to 5 ng o.c.). As expected, the use of larger injection volumes lowered the LOD and LOQ values for phosphate, sulfate, sodium, and potassium without adversely affecting the reproducibility data for these ions. Interestingly, neither nitrate nor chloride ions were quantified using the 50-µL injection

volume, due to significant solvent effects. Table IV lists the observed LOQ and LOD values, in nanograms on column, for each of the ions as well as the lowest observed LOD solution concentration, in ppb. All the values reported in Table IV are calculated in relation to specific ion concentrations and not that of the salt solution which was injected.

Method optimization and selectivity changes

It was determined that slight modifications to gradient method #1 would be required for some API counter-ion analysis. The ZIC–pHILIC gradient approach can easily be modified to alter the retention of the APIs and counter-ions. For example, this change could be used to delay elution of the API from the void. The APIs are affected primarily by the hydrophilic interactions of the HILIC, and therefore they are retained more with the lower aqueous concentration. This can be achieved by lowering the starting percentage of mobile phase B (50% aqueous buffer) from 45% to 20%. Minor changes to gradient method #1 enabled the resolution of the additional ions listed in Table V (also see Figure 3). These effects are the result of an increase in buffer strength between mobile phases A and B. This is as expected, because the retention of ions is primarily affected by the electrostatic interactions with the stationary phase, and this process can be correlated with buffer strength. Risely and Pack discussed that the retention of anions and cations could be inversely affected by buffer strength. Thus with gradient method #2, the greater buffer strength was associated with increased retention for anions. However, in this study, there was little effect with increased buffer strength on the retention time of cations. This result is possibly the consequence of increasing both the buffer strength and organic content of the mobile phase A during the gradient. Risely and Pack indicated that increased organic content resulted in longer retention and increased separation of sodium and chloride. They used an aqueous gradient to a final concentration of 90% aqueous buffer to achieve elution of all the ions. In this work, by varying the buffer concentrations between mobile phases and then adding additional organic modifiers (methanol and IPA), the improved gradient allowed for similar elution patterns with increased selectivity using a much lower final aqueous content. For method development, manipulation of the mobile phase composition can be readily used to affect analyte retention time.

Counter-ion recovery in APIs

The modified gradient method #2 was used to analyze several concentrations of common API counter-ions. Four-point standard curves were prepared between 18 and 28 µg/mL for chloride, bromide, sulfate, and sodium salts. Samples containing an API were prepared at concentrations which would have theoretical counter-ion values within the range of the calibration curve. The experimental counter-ion concentrations were then determined and these are listed along with the theoretical values in Table VI. The calculated concentrations were found to be within 95–105% of the theoretical counter-ion concentrations for all API samples evaluated. The relative retention times are also listed in Table VI for each of the APIs, along with the retention times for their associated counter-ion.

Detection of 0.1% impurities

Experiments were performed to determine whether a response could be observed for a 0.1% ion impurity in the API

Table IV. Limits of Detection and Quantification

Analyte	LOQ (ng O.C.)	LOD (ng O.C.)	Solution concentration
Nitrate	4	1.3	100 ppb*
Chloride	4	1.3	90 ppb*
Phosphate	12	7	150 ppb†
Sulfate	7	2.5	85 ppb†
Sodium	4	1.3	40 ppb†
Potassium	5	3	60 ppb†

* Maximum injection volume used 20 µL.
† Maximum injection volume used 50 µL.

Table V. Retention Times and Tailing Factors Using Gradient Methods #1 and #2

	Method #1		Method #2	
Analyte	Retention (min)	Tailing	Retention (min)	Tailing
Anions:				
Phosphite	4.99	1.21	8.22	1.22
Nitrate	5.88	1.16	7.30	1.15
Perchiorate	6.12	1.16	6.00	1.12
Chloride	6.42	1.17	9.36	0.86
Arsenate	7.07	1.17	13.11	1.17
Bromide	7.41	1.14	10.07	1.15
Phosphate	7.62	1.19	14.16	1.19
Iodide	7.64	1.08	8.97	1.09
Sulfate	13.40	1.31	22.19	1.27
Cations				
Lithium	12.23	1.09	14.66	1.09
Sodium	15.62	1.17	18.78	1.14
Potassium	18.30	1.17	21.15	1.13

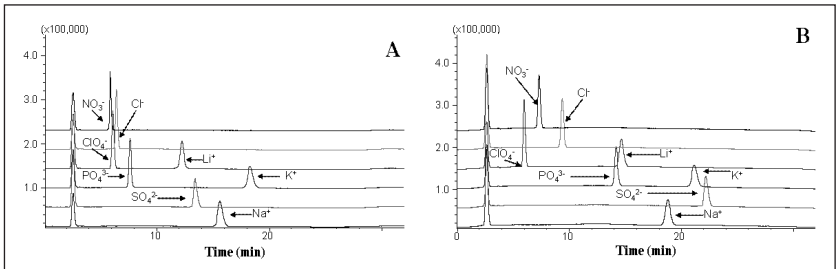

Figure 3. Overlays of anions and cations [10 µL injections of a 30 µg/mL (30 ppm) salt solution] analyzed using gradient method #1 (A). Improved resolution for overlays of anions and cations [10 µL injections of 30 µg/mL (30 ppm) salt solution] analyzed using gradient method #2 (B).

Table VI. Counter-Ion Comparison to Theoretical Values for APIs

APIs	Counter ion	k' (reative retention) API	k' (reative retention) Counter ion	Theoretical % Counter ion	Experimental % Counter ion
Verapamil hydrochloride	Cl^-	0.27	2.4	7.2	7.0
Procainamide hydrochloride	Cl^-	1.3	2.4	13	12.7
Dextromethorphan hydrobromide	Br^-	N/D	2.8	21.3	22.4
Quinine sulfate dihydrate	SO^{2-4}	0.86	7.0	12.3	11.9
Diclofenac sodium salt	Na^+	0.11	6.0	7.2	7.2

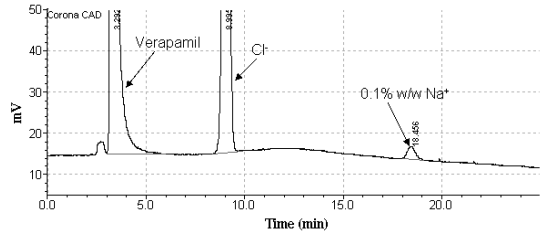

Figure 4. Expanded baseline for chromatograph (10 µL injection of 0.7 mg/mL) of verapamil HCL with a 0.1% w/w spike of sodium analyzed using gradient method #2.

sample. A sodium spike in a verapamil hydrochloride API solution and a chloride spike in a diclofenac sodium salt API solution were prepared as discussed in the "Experimental" section. The total concentrations of the solutions were 0.7 mg/mL of verapamil hydrochloride and 0.3 mg/mL diclofenac sodium salt for the respective sodium and chloride impurity responses. These spiked samples were analyzed using both the gradients, methods 1 and 2. In all cases, the impurity response of the spike was greater than the LOD for that ion using an S/N value > 3. Figure 4 shows the chromatograph of the verapamil sample with the baseline expanded to clearly show the impurity. The S/N for the ion impurity is clearly sufficient to achieve accurate and quantitative results at the 0.1% level or below. The uniform response characteristics along with the large dynamic range of the CAD allows for the API and the impurity to be analyzed in the same sample. This is in contrast to the ELSD work presented in the Risley and Pack where the ion standards were prepared at concentrations of 0.2–0.7 mg/mL, and the responses for the counter-ions were much lower than those observed for the APIs. Performing low-level impurity analysis at 0.1% of the API is a requirement in the pharmaceutical industry, and this would not be obtainable with an ELSD. The response of an ELSD does not contain sufficient dynamic range or sensitivity for this level of impurity analysis. A similar performance of the CAD was also observed for the analysis of ion impurities found in diclofenac sodium salt. In this case, the chloride impurity at 0.1% was easily detected using ZIC–pHILIC with CAD.

Conclusion

A flexible gradient method was developed for the simultaneous determination of anions and cations in a single analysis using common HPLC equipment and CAD. The HILIC–CAD approach is easily applied to the concurrent measurement of APIs and their counter-ions. Furthermore, the method is both sensitive (high ppb level without sample pre-concentration) and possesses a wide dynamic range, enabling the measurement of ionic impurities at the 0.1% level and below. The method is accurate, precise, and sensitive to the ppb range. The HILIC–CAD approach is a viable alternative to IC-conductivity detection for the measurement of ions using common HPLC components and yielding superior results to ELSD.

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