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Development and validation of a short runtime method for separation of trace amounts of 4-aminophenol, phenol, 3-nitrosalicylic acid and mesalamine by using HPLC system

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In a short runtime process (about 6 minutes), with isocratic method without need of reconditioning before the next run, a mixture of some molecules containing 4-aminophenol (4APh), phenol, 3-nitrosalicylic acid (3NSA), and mesalamine (5-aminosalicylic acid) (MLZ), were separated and detected by means of HPLC/UV-Vis system. The resolutions of the separated and sharp peaks referring to the mentioned compounds were at least more than 2, and the tailing factor (TF) was about 1, even in 1 µg/l (ppm) concentration. In order to optimize the method, the effects of the pH, eluent percentage, flow rate, and buffer concentration, and wavelength on the chromatogram were investigated. Also, the results of the validation processes showed that the method trustable to be used in laboratories.

1. Introduction

In one hand, separation and detection of trace amounts of phenolic or benzylic acid compounds are important both in medicinal (human metabolism)[1] and environmental chemistry,[2] and in the other hand, determination of one part per million (PPM or µg/l) concentration of some of the aminophenols in Mesalazine is crucial in pharmacology.[3] Due to these requirements, designing suitable methods for Related Impurity Analysis (RIA),[4,5] Assay Analysis (AA),[6] Residual Solvent Analysis (by gas chromatography) (RSA),[7] and other instrumental analysis for MLZ and related compounds were designed, and widely applied. Song and colleagues designed an electrochemical method for RIA of MLZ using a C8 column, with the mobile phase of PDP, and sodium 1-octanesulfonic acid sodium salt (OSA) buffer solution at pH 2.2, methanol, acetonitrile (890:80:30) and methanol via gradient elution. A glassy carbon electrode and a silver-silver chloride electrode were used for the electrochemical detection, respectively.[4] The pharmacopeias like UPS have proposed some related HPLC methods for RIA of MLZ using UV detectors.[7] Sometime during synthesis of organic compounds[8-10] a number of

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organic impurities like precursors, intermediates, or degrading products are formed that is, the RIA is very important for detection of probable impurities. An example for HPLC assay method was a chromatography condition containing a SuperPac Sephasil C18 column (5 µm;250 mm, 4.0 mm; SuperPac Sephasil). The samples were isocratically eluted with a 30 mM PDP buffer and methanol with a volume ratio of 70:30. The buffer was also contained 25% tetrabutylammonium hydrogen sulphate, with the flow rate of 1.0 mL/min. The UV detection at 254 nm was performed.[6]

In the case of choosing methods for use, when applying the gradient HPLC methods, after the end of the running process, the operator must consider a time for re-conditioning of the system at the zero point of elution (unlike isocratic methods).[11] Therefore, sometimes operators (especially in industries) and commercially users prefer the isocratic method that does not need for re-conditioning. But, use of isocratic methods may results in poor separation, low resolution of the peaks, and long running time.[12] Thus, in many cases, method designers have to develop gradient methods instead of isocratic ones. In an equal condition, when the runtime, resolutions, and other parameters both for gradient and isocratic methods are the same, the isocratic method maybe preferred especially in industries or in laboratories.[13] On the other hand, methods with the ability of separating mixed standards are more efficient and more preferred than methods which could separate only one or two standards. If a method could separate a mixed of a number of standards by a single run instead of preceding several runs for a number of single standards, there will be a great saving in time, energy, and solvent. Thus, designing methods which separate and detect a mixed of standards by a single run, with sharp, symmetric and high resolution peaks, would be useful and interested both for laboratories and industries.[14]

Due to above mentioned debates, in this research, we have designed a short (in 6 minutes), single run isocratic method that is able to separate 4-aminophenol (4APh), phenol, 3-nitrosalicylic acid (3NSA), and mesalamine (5-aminosalicylic acid) (MLZ) resulting in high resolution and sharp peaks. In the optimized chromatography condition, a C18, end-capped (250 × 4.6) mm, 5µm liquid chromatography column was used for the analysis. The mobile phase was a mixture of (buffer:methanol) with a ratio of (60:40). The buffer was prepared by dissolving 1.1 g of (5mM) OSA in 1 liter of water to pH=2.70 with OPA. The isocratic elusion with a flow rate of 1.5 ml/min was set for the method. In addition, a UV detector on the wavelength of 210 nm was used for recording the chromatograms. The column oven temperature was maintained at 40°C and the volume of injection was 20 µL. Also, the mobile phase was used as the diluent. The sample solutions were prepared as following.

2. Results and Discussion

In the present project, at the first step, several parameters were optimized to reach the best conditions and then, the limit of detection (LD), linearity, and some other validation parameters were investigated. Following sections describe the process of optimization and further investigations.

2.1. Effect of various parameters

In order to find the best chromatography condition with higher possible separation, resolution, and linearity, and also to reach to the lowest limit of detection, several parameters containing the concentration of PDP, and OSA salts, pH of the buffer, organic solvent percentage, flow rate, and wavelength were optimized. The results showed that by optimizing each of the mentioned parameters, higher resolution, and better separation could be reached.

2.1.1. Effect of PDP

At the beginning, we prepared a buffer by dissolving 10mM PDP (both as pH controller), and 10mM OSA as column modifier (for better interactions between the stationary phase and the components of the mixed standard) to different pHs optimized by OPA as the acid agent. The mobile

phase contained of 60% of buffer and 40% of methanol as organic solvent. Then, in the first step, we changed the concentration of PDP from 20mM, to 10mM, 5mM, 1mM, and finally elimination of the salt from above mentioned buffer. The results showed that decreasing the PDP salt in buffer or even elimination of that (it means just OSA is used), could result in better separation and higher resolution of all four peaks. While using that salt increase the tailing factor of the peaks. On the other hand, elimination of PDP and use of only the OSA salt, led the method to be easier and cheaper. Due, to these, the PDP was eliminated from buffer and all of the optimization processes were performed by OSA.

2.1.2. Effect of pH

The results showed that changes on the pH strongly effects on the chromatogram resolution, peak separation, and the RT of those. In order to optimize the pH value, we fixed all mentioned parameters and only changed the Ph. At the first step, the pH was set on about 5.5. At this pH, peaks 1, and 2 appeared about 1.7 min and 2.2 min respectively, while peaks 3, and 4 appeared in one peak. By decreasing the pH to 3.3, the RT of peaks 1, and 2 changed to about 1.8min and 2.4min, while the unique peak which represented peaks 3, and 4 broke in two overlaid peaks (Figure 1).

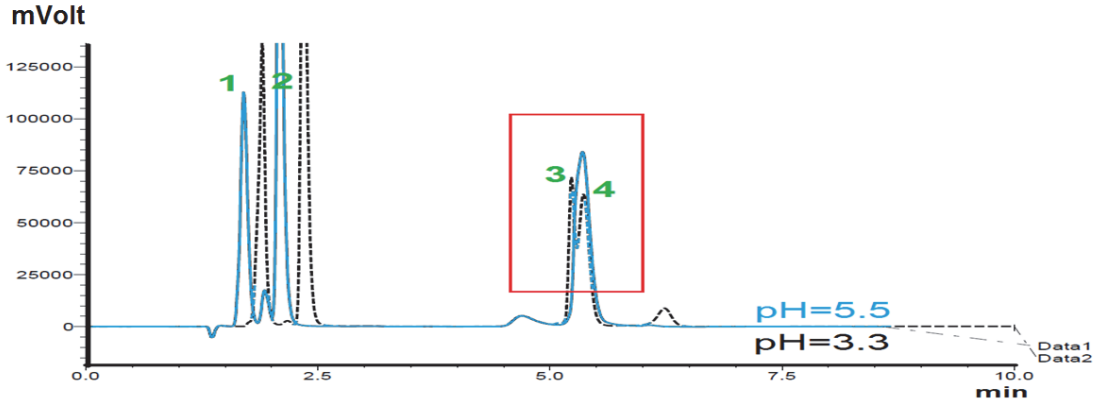

Fig. 1. The effect of pH=5.5, and pH=3.3 on the RT of peaks, and splitting between peaks 3, and 4.

In addition, we changed the pH to different values. Fig. 2 shows our results reached by pHs about 3.3, 2.7, and 2.5. The results showed that by decreasing the pH values from 3.3 to 2.5, the splitting between peaks 3, and 4 increase and also peaks 1, and 2 moves to upper RTs. Due to these, pH=2.7 was selected as the optimum pH, and in further experiments, other parameters were being optimized.

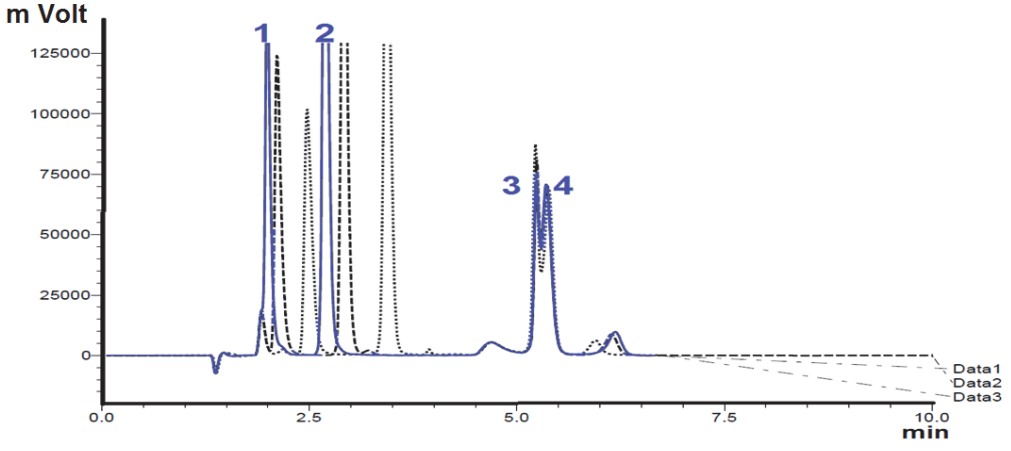

Fig. 2. Effect of pH on the RT of peaks, and splitting of peaks 3, and 4. Blue line, dashes, and dots, represent pH=3.3, pH=2.7, and pH=2.5.

2.1.3. Effect of methanol percentage

All of the liquid chromatography parameters were being fixed, and the percentage of methanol was the only parameter which was changed. As shown in Fig. 3, by changing the methanol percentage from 40% (blue line) to 45% (dash) and 50% (dots), the separation of the peaks 3, and 4, were significantly better; while, in the case of peaks 1, and 2, it resulted in overlaying of those two. Moreover, by increasing the methanol percentage to 50%, peak 1 was linked to the injection peak (red circle).

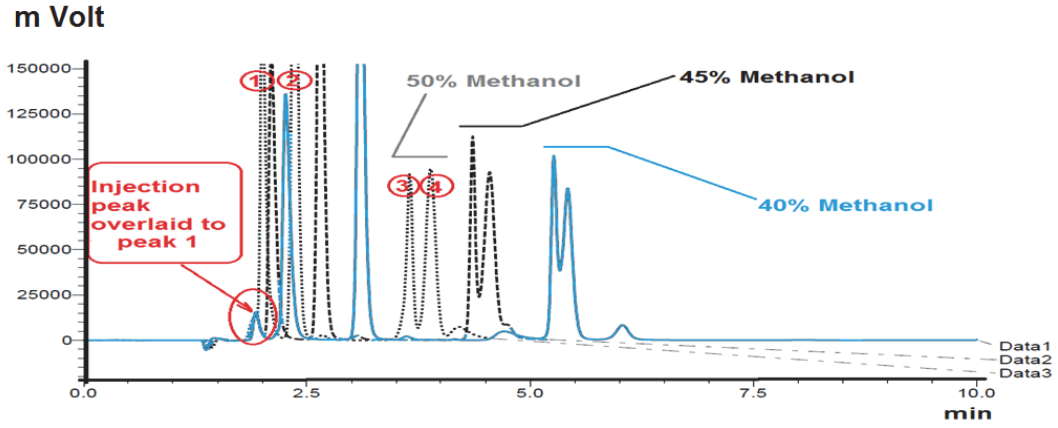

Fig. 3. Change of methanol concentration results in change of separation between peaks 3, and 4.

Also, another experiment showed that using 37% of methanol led to a very weak separation of peak 3, and 4 compared to 40%. In the following section, the experiments showed that by selecting the methanol percentage on 40%, and changing OSA concentration in buffer, the separation of peaks 3, and 4 became better, while it does not lead to a bad effect on peaks 1, and 2 separations (**Fig. 4**).

2.1.4. Effect of OSA concentration

As shown in **Fig. 4**, the concentration of OSA in buffer, directly effects on the peak separations and also on the position of those. Somehow, by using the 10mM of OSA in buffer (dots), peaks 1, and 2 are pretty separated, while peaks 3, and for (RT about 5.5 min) are overlaid. Also, by using 2.5 mM of OSA (blue line), peaks 3, and 4 are separated, but peaks 1, and 2 are overlaid. Then, we used 5mM of OSA in buffer (green dashes) and observed that both groups of peaks (peaks 1 and 2, and peaks 3, and 4) are separated. Thus, we choose 5mM of OSA in buffer concentration as the optimum for this parameter.

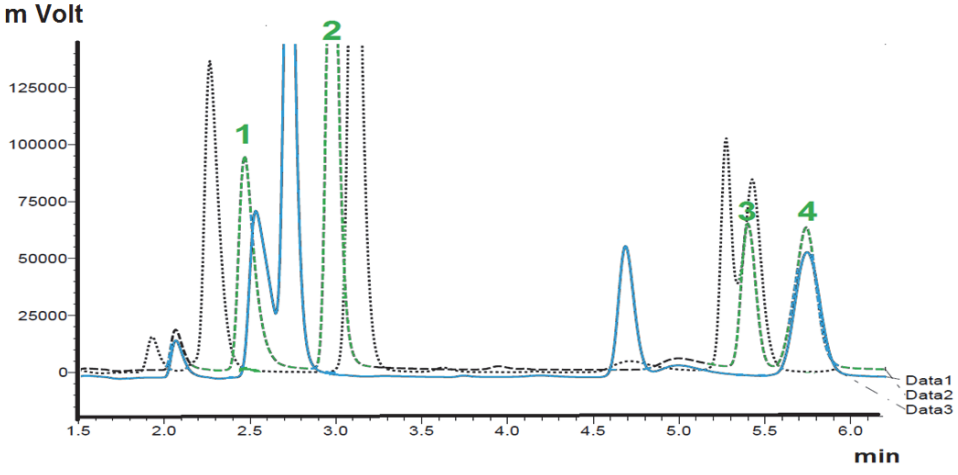

Fig. 4. The effect of OSA concentration in buffer solution, on the peak separation

2.1.5. Effect of flow rate change

As shown in **Fig. 5**, the flow rate changes from 1.5 ml/min to 1.3 ml/min showed that it just changes the RT of the peaks and it does not lead to a better separation of peaks 3, and 4 (4APh, and Phenol, respectively).

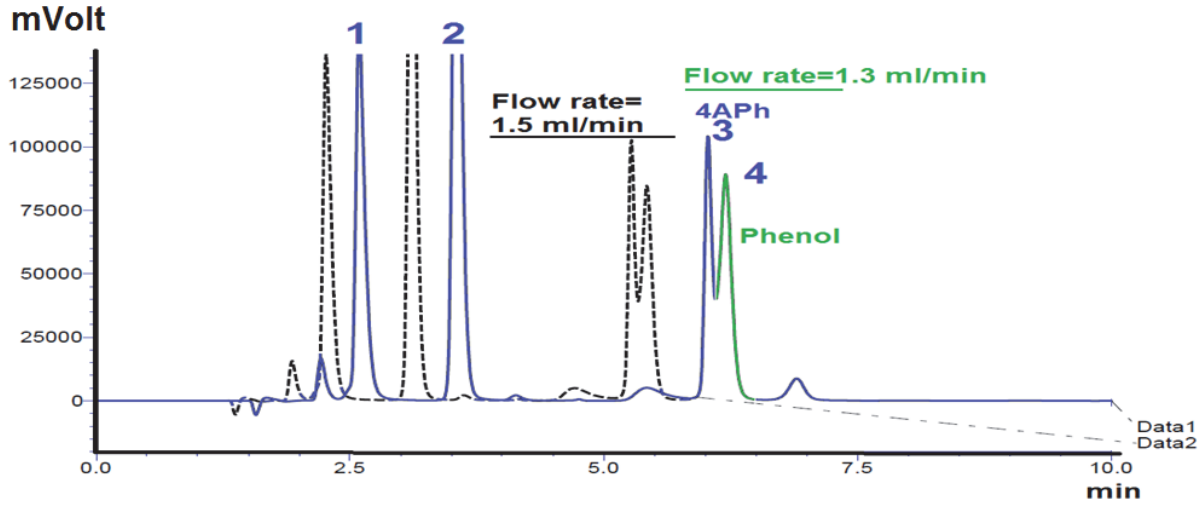

Fig. 5. The effect of flow rate change from 1.5 ml/min to 1.3 ml/min

2.1.6. Effect of the wavelength

As shown in **Fig. 6**, by decreasing the number of the wavelength from 240 nm to 210 nm, the intensity of the peaks increases. Somehow, at 210nm, all four peaks could be detected strongly. At 220 nm, the area of peaks 1, and 2 (RT=2.5, and 3) significantly decrease, while it is not observed for peaks 3, and 4 (RT=5.4, and 5.7min). in addition, at 230nm, and 240nm, a considerable decrease is observed for all peaks. It is notable that at 240 nm, peaks 3 and 4, could not be detected in practice. Since at wavelengths lower than 210nm, the UV cutoff of elution may affect on the efficiency of the optimized method, the 210nm was selected as the optimum wavelength.

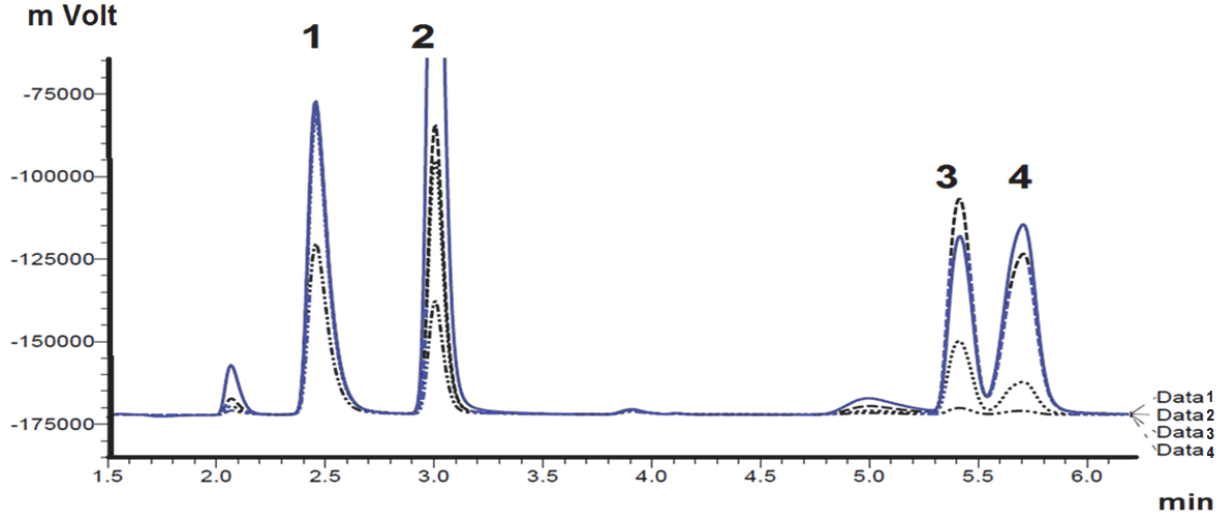

Fig. 6. The effect of the recording wavelength, on the peak intensity (mV.min). The blue gram (Data 1) is 210nm, and the Data 2, Data 3, and Data 4, are 220nm, 230 nm, and 240 nm, respectively.

2.2. Validation of the optimized method

In the optimized chromatography condition, a C18, end-capped (250 × 4.6) mm, 5µm liquid chromatography column was applied for the solid-liquid phase chromatography separation. The buffer was prepared by dissolving 5mM octane-1-sulfunic acid sodium salt in water to pH=2.70 by OPA (then filtered by a 0.45µm filter). The mobile phase was a mixture of (buffer/methanol) with a ratio of (60/40). The isocratic elution was operated at a flow rate of 1.5 ml/min. The oven temperature was about 25°C, and the chromatograms were recorded on 210nm. By setting these parameters, all four peaks referring to 3-nitrosalicylic acid, mesalamine, 4-aminophenol, and phenol (respectively), were separated and detected with a low tailing factor and a good resolution.

2.2.1. Linearity study

It is obvious that the linearity of an analytical method is its ability (within a considered range) to give results which are directly (or by a mathematical transformation), proportional to the concentration of the sample (or standard). The linearity of the present procedure was assessed by injecting the solutions of a mixed standard containing four related compounds, listed in **Table 1** (each solution was injected three times). A linear calibration curve was prepared by plotting the average analytical signal versus mixed standard concentration (**Fig. 7**). A regression line was calculated by the procedure of least squares.

Table 1. The areas (mV.min) of four peaks referred to 3NSA, MLZ, 4APh, and Phenol, respectively, obtained by recording the mixed standard solutions from 0.1ppm to 10ppm

Concentration	3NSA	MLZ	4APh	Phenol
0.01 ppm	220	1237	-	-
0.1 ppm	4278	11779	2269	9977
0.5 ppm	24657	63664	16253	30417
1 ppm	58478	123849	34800	57494
5 ppm	299201	589663	181401	274698
10 ppm	626286	1190157	380162	564115
R^2	0.999	0.999	0.999	0.999

The linearity curve given in **Fig. 7**, indicates that the coefficient of determination (R^2) for each of four standards was 0.999 which showed a good linearity in the concentration range of 0.1 ppm to 10 ppm. Also, in the case of MLZ, the concentration ranges begin from 0.01 ppm to 10 ppm.

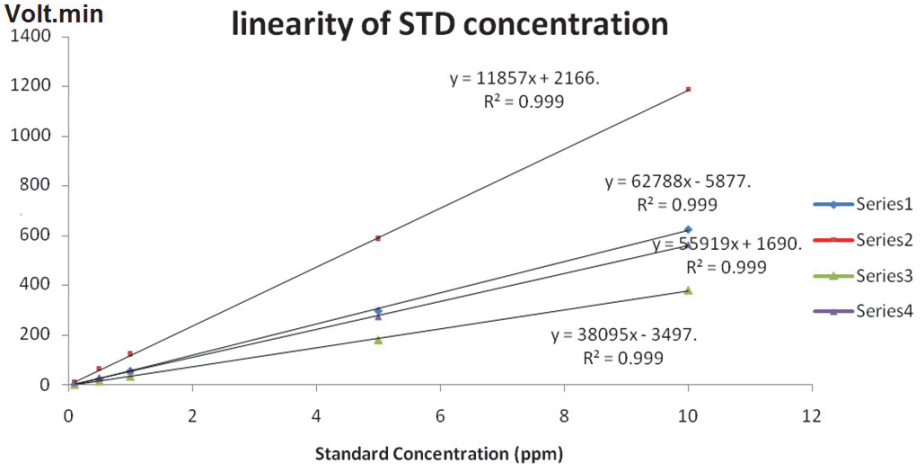

Fig. 7. The calibration curve for the linearity study referred to four considered standards. The series 1 to 4, represent 3NSA, MLZ, 4APh, and Phenol, respectively.

2.2.2. Limit of Detection (LD) and Quantification limit (QL)

As given in literature, the limit of detection (LD) of an individual analytical method is the lowest possible amount of analyte in a sample which could be detected; while, it is not necessarily quantitated as an exact value. Moreover, the limit of quantitation (QL) of an analytical approach is the lowest amount of analyte in a sample, which could be quantitatively determined with a suitable level of precision and accuracy. As given in following, in this study, LD and QL were determined based on the standard deviation of the y-intercept and the slope of the calibration curve.[15]
The LD and QL are expressed as (Eq. (1), and Eq. (2)):

$$LOD = \frac{3.3\,\sigma}{S}, \quad (1)$$

$$LOQ = \frac{10\,\sigma}{S}, \quad (2)$$

where, σ is the standard deviation of y-intercept, and S is the slope of the calibration curve. The LD and QL values are presented in Table 3.

Table 2. LD and QL values of the method for mixed standards calculated by Eq. (1), and Eq. (2).

	3NSA	MLZ	4APh	Phenol
Slope	62788	118571	38095	55919
Standard deviation of y-intercept	5877.6	2166.6	3497.3	1690.6
LD (mg/L)	0.309	0.060	0.303	0.100
QL (mg/L)	0.936	0.183	0.918	0.302

The results showed that the (LD; QL) values for 3NSA, MLZ, 4APh, and Phenol, are (0.309;0.936), (0.060;0.183), (0.303;0.918), and (0.100;0.302), respectively; which indicate the method is properly sensitive. The acceptance limit for LD and QL is 0.5 mg/L, 1.0 mg/L, respectively.

2.2.3. Accuracy

The accuracy of the method has been evaluated by means of analyzing mixed standard concentration levels. The recovery percentages were calculated regarding to the regression equation. The results show that the recovery data are in the acceptable range of 99.9-100.2%. Accuracy is commonly reported as the percent recovery of the known, added amount, or as the difference between the mean and true value with confidence intervals. The acceptance limit for recovery data are from 99.5-100.5%.

Table 3. The accuracy of the method by using the recovery data at 5 ppm

	3NSA	MLZ	4APh	Phenol
Slope	62788	118571	38095	55919
Standard deviation of y-intercept	5877.6	2166.6	3497.3	1690.6
Area (based on Calibration curve)	299201	589663	181401	274698
Recovery%	99.9	100.0	100.2	99.9

2.2.4. Precision

Due to the literature, the precision of an analytical method is based on the closeness of agreement between a series of measurements prepared from multiple sampling of the same homogeneous sample under a defined condition. Thus, the precision is the standard deviation, variance, or coefficient of variation of a series of measurements.[16]

2.2.5. Repeatability

Repeatability of the present method was assessed by analyzing the mixed standards at 100% of the test concentration. As given in Table 4, the % RSD values for peak responses are less than 2.0% which could be acceptable. The acceptance limit for %RSD is 2.0%.

Table 4. The interday, and the intraday precision for the mixed standards

	3NSA	MLZ	4APh	Phenol
First day (Run 1)	619024	1180134	371968	560698
First day (Run 2)	624240	1187175	373721	563044
First day (Run 3)	621083	1195911	373165	562822
Second day (Run 1)	626286	1190157	380162	564115
Second day (Run 2)	627146	1180734	377869	566153
Second day (Run 3)	621165	1182584	373677	562913
Average (First day)	621449	1187740	372951	562188
Average (Second day)	624866	1184492	377236	564394
RSD (First day)	0.42	0.67	0.24	0.23
RSD (Second day)	0.52	0.56	0.43	0.25
RSD (Intraday)	0.58	0.58	0.58	0.58

3. Conclusions

In this project, a reverse phase HPLC method for a very fast (about 6 minutes) and simple separation of mesalamine from 4-aminophenol, phenol, and 3-nitrosalicylic acid was developed. The chromatogram peaks refereed to the four mentioned compounds were mostly high resolution. The results showed that 0.01µg/l (0.01 ppm) of mesalamine could be detected by a sharp-tiny peak, which shows the sensitivity of the method in detection of this compound. The method was shown to be robust within the defined design conditions. A cheap and usual C18 liquid chromatography column was used for the process, and the results showed that the mentioned column was suitable for the high-resolution separation of the considered compounds. The results showed that by increasing the methanol percentage, separation between peaks 3, and 4, increases; while peaks 1, and 2 become closer to each other and to the injection peak. Also, changing the pH of the buffer showed that it has significant effects on the peak separation, resolution, and retention times. It is probably due to amphoteric nature of all four considered compounds. Especially in the case of peaks 3, and 4, by decreasing the pH from about 5.5 to 2.5, separation between those two has a great and observable increase. The linearity curve indicated that the coefficient of determination (R^2) for each of four standards were 0.999 which showed a good linearity in the concentration range of 0.1 ppm to 10 ppm. Also, the results showed that the (LD;QL) values for 3NSA, MLZ, 4APh, and Phenol, are (0.309;0.936), (0.060;0.183), (0.303;0.918), and (0.100;0.302), respectively; which indicate the method is properly sensitive.

Supplementary Data

Supplementary data are available at the Journal home page.

Acknowledgment

Authors would like to thank Tofigh Daru Research and Engineering Company for financial and technical support of this work.

4. Experimental

Chemicals containing PDP, ortho-phosphoric acid (OPA), methanol, octane-1-sulfunic acid sodium salt (OSA), 4-Aminophenol (4APh), and phenol were prepared from Merck chemical company (Germany). Mesalamine (MLZ) and 3-nitrosalicylic acid (3NSA), compound was provided from the Chemical Synthesis Department of Tofigh Daru Research and Engineering Company (Tehran, Iran).

4.1. Instrumentation

The Shimadzu Prominence system (Shimadzu Corporation, Kyoto, Japan) equipped with a LC-20AD pump, a DGU-20A degassing system, a CTO-20A column oven and a SPD-20A UV-Vis

detector, was used for all analysis. Also, the LabSolutions software version 5.51 was applied for processing and data analysis.

4.2. Chromatographic conditions and sample preparation

In the optimized chromatography condition, a C18, end-capped (250 × 4.6) mm, 5µm liquid chromatography column was used for the analysis. The mobile phase was a mixture of (buffer:methanol) with a ratio of (60:40). The buffer was prepared by dissolving 1.1 g of (5mM) OSA in 1 liter of water to pH=2.70 with OPA. The isocratic elusion with a flow rate of 1.5 ml/min was set for the method. In addition, a UV detector on the wavelength of 210 nm was used for recording the chromatograms. The column oven temperature was maintained at 40°C and the volume of injection was 20 µL. Also, the mobile phase was used as the diluent. The sample solutions were prepared as following:

Test solution (a:1000ppm): Dissolve 10 mg of each of the considered substances containing 3NSA, MLZ, 4APh, and Phenol the diluent (1000 mg/L).
Test solution (b:10ppm): Dilute 1.0 mL of Test solution (a) to 100.0 mL with the diluent (10 mg/L).
Test solution (c:5ppm): Dilute 5.0 mL of Test solution (b) to 10.0 mL with the diluent (5 mg/L).
Test solution (d:1ppm): Dilute 2.0 mL of Test solution (c) to 10.0 mL with the diluent (1 mg/L).
Test solution (e:0.5ppm): Dilute 5.0 mL of Test solution (d) to 10.0 mL with the diluent (0.5 mg/L).
Test solution (f:0.1ppm): Dilute 2.0 mL of Test solution (e) to 10.0 mL with the diluent (0.1 mg/L).
Test solution (g:0.01ppm): Dilute 1.0 mL of Test solution (f) to 10.0 mL with the diluent (0.01 mg/L).
The injections were 20 µL of the Blank solution (which is the mobile phase, used as the diluent), the Test solutions (a) to (g), respectively.

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