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QUANTITATIVE DETERMINATION OF RESIDUAL PHOSPHATE AND PHOSPHITE IN BISPHOSPHONATES BY ION EXCHANGE CHROMATOGRAPHY USING CONDUCTIVITY DETECTION

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ABSTRACT

The present paper deals with the development and validation of analytical method based on a single column high performance Ion chromatography with cation suppressed conductivity detection, developed for quantitation of residual phosphate and phosphite impurities. The diluent used for the preparation of sample solution was water : Acetonitrile (80:20 v/v) and injected into a standard chromatographic system connected with 250mm length, 4.0 mm ID and 5.0 µm particle size Metrosep A supp 5 Ion exchange column and suppressed conductivity detector. The degradation studies employed with all the drug substances namely Alendronate sodium, Pamidronate disodium, Risedronate sodium and Zoledronate disodium salts show a stability indicating method and not affected by degradation impurities. The developed analytical method was highly sensitive to detect the phosphate and phosphite, shows limit of detection and limit of quantitation as 30 ng/ml and 100 ng/ml respectively. Calibration curves were linear with correlation co-efficient of > 0.999 for both phosphate and phosphite respectively. The % RSD of area response of phosphate and phosphite in standard injections was below 2.0, which demonstrates the system precision. The % RSD of results in samples is below 2.0, which demonstrates the method precision of the test procedure. The accuracy of the method is also studied for all four bisphosphonates, observed between 90% and 110%. The developed method was fully validated according to ICH guidelines for the quantitative determination of residual phosphite and phosphate in all four bisphosphonates taken for the study.

Keywords: Ion chromatography, Conductivity detector, Phosphate, Phosphite, Bisphosphonates, Alendronate sodium, Pamidronate disodium, Risedronate sodium, Zoledronate disodium

INTRODUCTION

Bisphosphonates were developed in the 19th century, but were first investigated in 1960's for the use in disorder of bone metabolism. They are a class of compounds which share a common P-C-P backbone in their structure. The initial rationale for the use in humans was their potential in preventing the dissolution of hydroxyl apatite, the principle bone mineral, and hence arresting the bone loss[1]. Bisphosphonates are used clinically for the treatment of Osteoporosis, Osteitis deformans (Paget's disease of the bone), bone metastasis (with or without hypercalcemia), multiple myeloma and other conditions that feature bone fragility. In Osteophoresis and Paget's disease, Alendronate and Residronate are the most popular first-line drugs[2]. High potency intravenous bisphosphonates have shown to modify progression of skeletal metastasis in several forms of cancer, especially breast cancer[3,4]. Phosphates and Phosphites are the important compounds with biological and chemical significance. These ions are also chemically

interested in that they form oligomeric species that means they exist in several oxidation states and can undergo multiple protonation and metal ligand reactions.

Several methods may be studied by a variety of analytical techniques for the determination of phosphate and phosphite from Coulometric detection to Capillary electrophoresis, LC-ESI-MS[5] and ^{31}P NMR analysis. However LC-ESI-MS, Capillary electrophoresis[6], and fluorescence detection with post column derivatization[7-11] are rarely used analytical techniques and may not be available in all quality control laboratories. Since the phosphate and phosphite ions were non-absorbing sample ions showing very low intensity and high detection limit in UV detector[4,12] (0.02% with respect to analyte concentration 1 mg/ml), the analytical method developed by ion exchange chromatography coupled with conductivity detector is relatively sensitive to detect the phosphate and phosphite impurities at optimum costs and it can be easily implemented in quality control laboratories for routine testing and release.

EXPERIMENTAL

Chemicals and Reagents:

Alendronate sodium chemically described as (4-amino-1-hydroxybutylidene) bisphosphonic acid monosodium salt trihydrate, Pamidronate disodium described as (3-amino-1-hydroxy-1-phosphono-propyl) phosphonic acid disodium pentahydrate, Risedronate sodium chemically described as ((1-hydroxy-1-phosphono-2-pyridin-3-yl-ethyl)phosphonic acid monosodium salt), Zoledronate disodium chemically described as (1-hydroxy-1-phosphono-2-imidazol-1-yl-ethyl) phosphonic acid disodium salt. Samples of these four compounds were received from Process Research Department of Custom Pharmaceutical Services, Dr. Reddy's Laboratories Limited, Hyderabad, India. The chemical structures of the above mentioned drug substances are shown in Figures.1, 2, 3 and 4.

Analytical grade sodium carbonate, sodium hydrogen carbonate was purchased from S.d.fine chemicals, Mumbai, India. and Analytical grade sodium hydroxide and disodium hydrogen phosphate from Qualigens Fine Chemicals, Mumbai, India. Analytical reagent grade sulfuric acid was purchased from Merck, Mumbai, India and phosphorus acid from Spectrochem, Mumbai, India. HPLC grade water used for the analysis was purchased from Qualigens Fine Chemicals, Mumbai, India.

Equipment:

The Ion Chromatography system purchased from Metrohm, Herisau, Switzerland used through out this study is equipped with 818 IC pump , 833 Liquid Handling unit, Sample injector with 20µL loop, 820 IC Separation center equipped with a Cation suppressor and Conductivity detector. Quantitation was performed from the output signal, monitored and processed using the IC Net 2.3 SR4 version software on Compaq computer (Digital Equipment Co). Dilutions were accomplished with Hamilton precision pipettes (Bonaduz, Switzerland).

Chromatographic conditions:

The chromatographic column used was a Metrosep A Supp 5 column (250 x 4.0 mm, 5.0µm Particle size) having stationary phase of Polyvinyl alcohol with Quaternary Ammonium groups, that was safeguarded with Metrosep A Supp 4/5 guard column. The mobile phase used was a mixture of 0.9 mM sodium hydrogen carbonate, 3.0 mM sodium carbonate and 4.0 mM Sodium hydroxide prepared in HPLC grade water, degassed and filtered. The flow rate of the mobile phase was set at 0.8 ml/min. The injection volume was 20 µL. Water: Acetonitrile (80:20, v/v) mixture used as a diluent.

The Anion exchange chromatographic system is equipped by a cation exchange resin suppressor for chemical suppression. Chemical suppression reduces the background conductivity and replaces the counter ions in the sample i.e all cations from the mobile phase are replaced by H^{+}. By this suppression reaction, an eluent with high conductivity is transferred to water and carbon di-oxide which is of low conductivity. Suppressor is regenerated after each run using a suppressor regenerator followed with suppressor rinsing with HPLC grade water. Suppressor regenerator used is 50 mM Sulphuric acid prepared in HPLC grade water. The detector interface was set with detector range 100 µS/cm and detector full scale 20 µS/cm. The run time for each run is 35 min.

Preparation of solutions:

The solutions of 1000 ng/ml and 100 ng/ml concentration of phosphate were prepared directly from 100 µg/ml stock solution of anhydrous di-sodium hydrogen phosphate. The solutions of 1000 ng/ml and 100

ng/ml concentration of phosphite were prepared directly from 100 µg/ml stock solution of Phosphorous acid. The test solutions are prepared by dissolving each bisphosphonate separately at the concentration of 1 mg/ml.

Method validation:

The objective of this work was to quantitatively determine the content of residual phosphate and phosphite impurities present in Bisphosphonates. Since most of the synthetic routes for preparation of Bisphosphonates were involved from alkyl phosphates and phosphites, the residual phosphate and phosphite were considered to be the potential impurities of Bisphosphonates. During method optimization, all chromatographic parameters were found to enhance precision, accuracy, limit of detection and limit of quantitation of phosphate and phosphite ion peaks, with appropriate analysis run time. All calculations concerning the quantitative analysis were performed with external standardization by measurement of the peak areas.

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities[13]. To demonstrate the proposed Ion exchange chromatographic method as a stability indicating procedure for Bisphosphonates, samples of Alendronate, Pamidronate, Risedronate and Zoledronate were subjected to forced degradation in acid, alkaline, photolysis, thermal and oxidative conditions. In the optimized ion exchange chromatographic system, the sample solution was injected after subjecting to the stress degradation with strong acid (0.1M Hydrochloric acid for 72 h), strong base (0.5 N sodium hydroxide for 72 h), oxidative degradation with 5% hydrogen peroxide for 5 h, thermal degradation at 105 °C for 72 h and photolytic degradation done by exposing the sample to the 1.2 million lux hours of UV light as per ICH guidelines[15].

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements from multiple sampling of the homogeneous sample under prescribed conditions[15]. The system precision for phosphate and phosphite was checked at 1000 ng/ml (0.1% level with respect to analyte concentration) and the percentage relative standard deviation of area for each peak was calculated individually. Method precision was checked for six different preparations of test samples of all the four Bisphosphonates. Each solution was prepared at the analyte concentration of 1 mg/ml. The percentage relative standard deviation of content of each impurity in six preparations was calculated. The intermediate precision of the method was also evaluated by different analyst and on different day. Each solution was prepared at the analyte concentration of 1 mg/ml.

The linearity of an analytical test procedure is its ability to obtain test results (within a given range), which is directly proportional to the concentration of the analyte in the sample[15]. The linearity of the method was checked at six different concentration levels from 100 ng/ml (LOQ concentration) to 1250 ng/ml of phosphate and phosphite individually. Each linearity solution was injected in duplicate and the average area response was used for plotting calibration curve. The calibration curve was drawn by plotting the peak areas of phosphate and phosphite impurities against corresponding concentration. The correlation coefficient of the regression line of the calibration curve is also calculated.

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the expected value found[15]. Standard addition and recovery experiments were conducted to determine accuracy of the quantitation of phosphate and phosphite in bulk drug samples of Alendronate, Pamidronate, Risedronate and Zoledronate. The study was carried out by addition of phosphate and phosphite at 500 ng/ml , 1000 ng/ml and 1500 ng/ml concentrations and injected to the chromatographic system in triplicate at each level. The % recoveries for phosphate and phosphite were calculated from the slope and y-intercept of the calibration curve obtained for all the four Bisphosphonates individually.

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated as an exact value[15]. The limit of detection for phosphate and phosphite was established by injecting a series of dilute solutions with known concentration [6] and the signal-to-noise ratio for peak response of phosphate and phosphite peak was calculated. The limit of detection was established at signal to noise ratio 3:1.

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy[15]. Limit of quantitation was estimated at signal to noise ratio 10:1. LOQ Precision study was also carried at the established concentration by injecting six individual preparations of phosphate and phosphite standards at LOQ level and the %RSD of the area was calculated, which was observed as less than 15.0.

The solution stability of all the Bisphosphonates was carried out by keeping both the test solutions of sample and reference standard in tightly capped volumetric flasks at room temperature for 48 hours. The same sample solutions were analyzed at twelve hours interval up to the study period. Further, mobile phase stability was also carried out for two days by analyzing the freshly prepared sample solutions against freshly prepared reference standard solutions for six hours interval. Mobile phase prepared was kept constant during the study.

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The flow rate of the mobile phase was 0.8 ml/ min in the method. To study the effect of flow rate on resolution, it was changed by 0.1 units to 0.7 ml/ min and 0.9 ml/min while the mobile phase components were held constant and the effect of flow rate was studied.

Robustness was not studied for the column temperature as the method employs the column equilibration at room temperature in analytical laboratory. The mobile phase used was highly basic due to presence of carbonates and hydroxide ions, so the difference of 0.2 pH unit was not considered appropriate to study the robustness.

RESULTS AND DISCUSSION

During specificity study, no significant variations were observed to the quality control samples for phosphate and phosphite peak estimation. However, oxidative treatment of Bisphosphonates shows additional peaks in different retention time with out disturbing the impurity profile of the standard. The oxidative degradation may cleave the organic moiety from the Bisphosphonates but did not show any major interference in phosphate and phosphite estimation [Fig. 5].

The system precision of the analytical method for phosphate and phosphite impurity was checked at the level of 1000 ng/ml (0.1% with respect to analyte concentration). The % RSD of the results was found to be 1.6 and 1.9 respectively for phosphite and phosphate that confirms good system precision. The method precision for the analytical procedure was checked with the six different test preparations of each bisphosphonate compound and %RSD of the results were tabulated [Table 1]. The intermediate precision also performed by the different analysts shows the % RSD less than 2.0 between the two analyst's results and inter-day analysis shows % RSD less than 2.0 [Table 2].

The limit of detection and limit of quantitation concentrations were found to be 30 ng/ml and 100 ng/ml respectively for both phosphate and phosphite peak, where signal to noise ratio of 3:1 for LOD (fig 6) and 10:1 for LOQ were used as a criteria, for 20 µL injection volume. Precision study was also carried out at LOQ level by injecting six individual preparations of phosphite and phosphate and % RSD for area response is 1.4 and 1.9 respectively.

Linearity graph was plotted over the range of concentration from 100 ng/ml to 1250 ng/ml. Six different preparation of phosphate and phosphite at 100 ng/ml, 250 ng/ml, 500 ng/ml, 750 ng/ml, 1000 ng/ml and 1250 ng/ml were injected The calibration curve is plotted against concentration and area response of the peak. The correlation coefficient (R^2) for the phosphate peak was 0.9991 having regression equation $y = 1.5436x-3.7706$. The correlation coefficient (R^2) for the phosphite peak was 0.9997 having regression equation $y = 2.1422x-1.9286$ [Table 3].

The proposed method was evaluated for recovery and estimated by the standard addition of phosphate and phosphite standards to Alendronate, Pamidronate, Risedronate and Zoledronate samples. The % recovery was calculated by actual area subtracting the amount of standard addition of phosphate and phosphite to the Bisphosphonates. The results are tabulated [Table 4 and 5] shows that the method performance is very good with the recovery of 90% to 110%.

No significant change was observed in sample analysis of Bisphosphonates even after 48 h during the solution stability and mobile phase stability experiments. Hence, the method is proven to be stability

indicating for the sample preparation and mobile phase system.The flow rate of the mobile phase was deliberately changed to 0.7 ml/min and 0.9 ml/min, while the mobile phase components were held constant and chromatograms were recorded for standard injection. The resolution between phosphate and phosphate peaks were calculated by the following formula,

Resolution = $2(t_2-t_1)/ w_1+w_2$

Where, t_1, t_2 = retention time of Phosphite and Phosphate ions

w_1, w_2 = the tangent peak widths of the Phosphite and Phosphate.

The resolution obtained were 12.62 and 12.01 for 0.7 ml/min and 0.9 ml/min flow rate respectively, which is almost equal to the standard resolution

A simple, sensitive and accurate analytical procedure was developed with ion exchange high performance liquid chromatography with conductivity detection which enables the determination and quantitation of residual phosphate and phosphite ions in Bisphosphonates with simple standard and sample preparation at optimum costs.

Satisfactory separation of phosphate and phosphite was achieved with the mobile phase system. The use of 4.0 mM sodium hydroxide in the mobile phase system helped to resolve the phosphate and phosphite peaks with the resolution of 12.30 [fig 7]. The validation of analytical method also shows the satisfactory data for the tested parameters. The developed method is stability-indicating and the phosphate and phosphite peaks are well resolved from with the degraded impurities. The method makes analysis procedure to be completed in shorter analysis time with good recovery, precision and sensitivity. The limit of detection of phosphate and phosphite also showed excellent results of 30 ng/ml and 100 ng/ml respectively. LOD concentration obtained by the proposed method was lower. Compared to other analytical methods, the results showed that the proposed method is highly accurate, precise and robust.

The advantage of this method is that it can be applied for all the four Bisphosphonates namely Alendronate sodium, Pamidronate disodium, Risedronate sodium and Zoledronate disodium, which was demonstrated in detail. On the basis of these results, the method is concluded to be suitable for quality control of phosphate and phosphite impurities in Bisphosphonates drug substances.

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Table-1 : Results obtained for method precision analysis (Test concentration 1 mg/ml)

Bulk drug	%RSD of 6 determinations	
	Phosphite	**Phosphate**
Alendronate	1.1	1.2
Pamidronate	0.8	1.3
Zoledronate	0.7	1.5
Risedronate	0.9	1.0

Table-2 : Intermediate precision: Inter-day testing of bulk drug samples (Test concentration 1 mg/ml)

Bulk drug	%RSD of 12 determinations	
	Phosphite	**Phosphate**
Alendronate	0.9	1.3
Pamidronate	1.1	1.2
Zoledronate	1.1	1.2
Risedronate	1.4	1.0

Table-3 : Linearity

Species	Line equation	R^2	Range**	LOD*	LOQ*
Phosphate	y = 1.5436x-3.7706	0.9991	0.1 – 1.25	30	100
Phosphite	y = 2.1422x-1.9286	0.9997	0.1-1.25	30	100

* Expressed in ng/ml
** Expressed in µg/ml

Table-4 : Summary of recovery experiments for phosphite peak

Drug substance	Added(µg)	Recovered	%Recovery	%RSD
Alendronate	0.519	0.489	94.2	7.9
	1.009	0.967	95.8	6.3
	1.542	1.489	96.6	8.8
Pamidronate	0.501	0.472	94.2	7.2
	1.012	0.967	95.8	6.8
	1.533	1.489	96.6	7.5
Zoledronate	0.498	0.469	94.3	7.7
	1.023	0.977	95.5	6.9
	1.508	1.449	96.1	8.1
Risedronate	0.51	0.474	92.9	6.3
	1.000	0.948	94.8	5.5
	1.503	1.45	96.5	6.1

n=3 determinations

Table 5 : Summary of recovery experiments for phosphate peak

Drug substance	Added(µg)	Recovered	%Recovery	%RSD
Alendronate	0.504	0.464	95	5.5
	1.005	0.958	95.5	6.9
	1.550	1.490	96.3	7.2
Pamidronate	0.499	0.46	92.3	6.6
	1.010	0.954	94.5	5.6
	1.525	1.452	95.2	6.8
Zoledronate	0.502	0.467	93.1	8.8
	1.091	0.104	95.3	6.2
	1.511	1.452	96.1	5.2
Risedronate	0.497	0.461	92.8	9.0
	1.013	0.955	94.3	6.7
	1.528	1.461	95.6	5.5

n=3 determinations

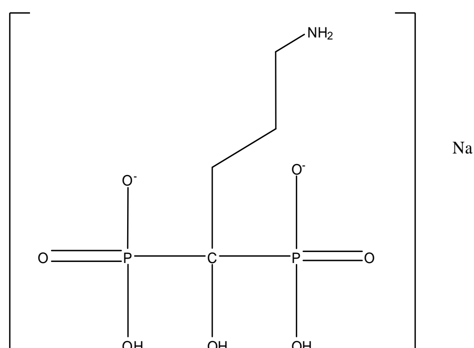

Fig 1: Alendronate sodium

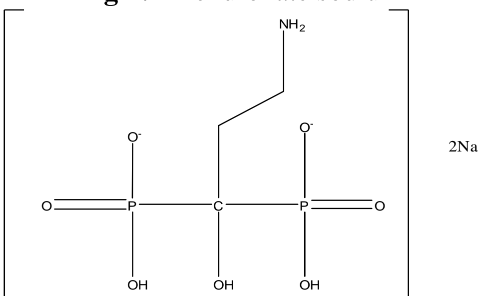

Fig 2: Pamidronate disodium

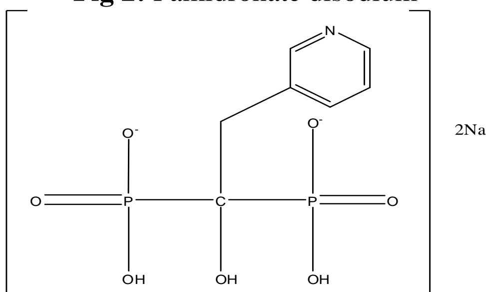

Fig 3: Risedronate disodium

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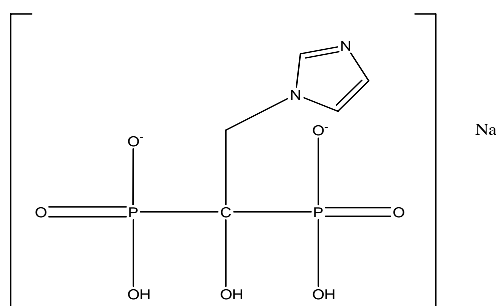

Fig 4: Zoledronate Sodium

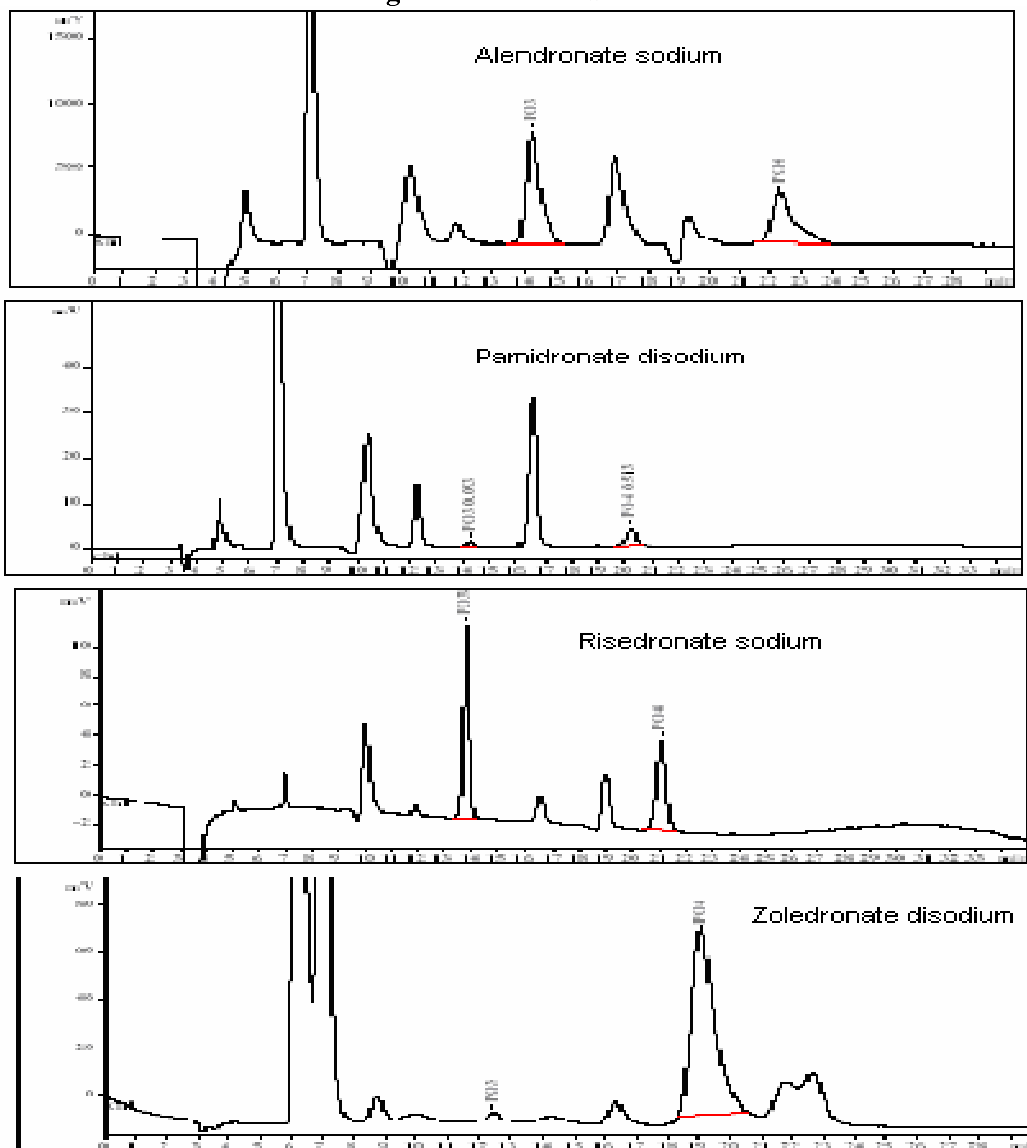

Fig 5: Chromatogram of oxidative degradation of bisphosphonates

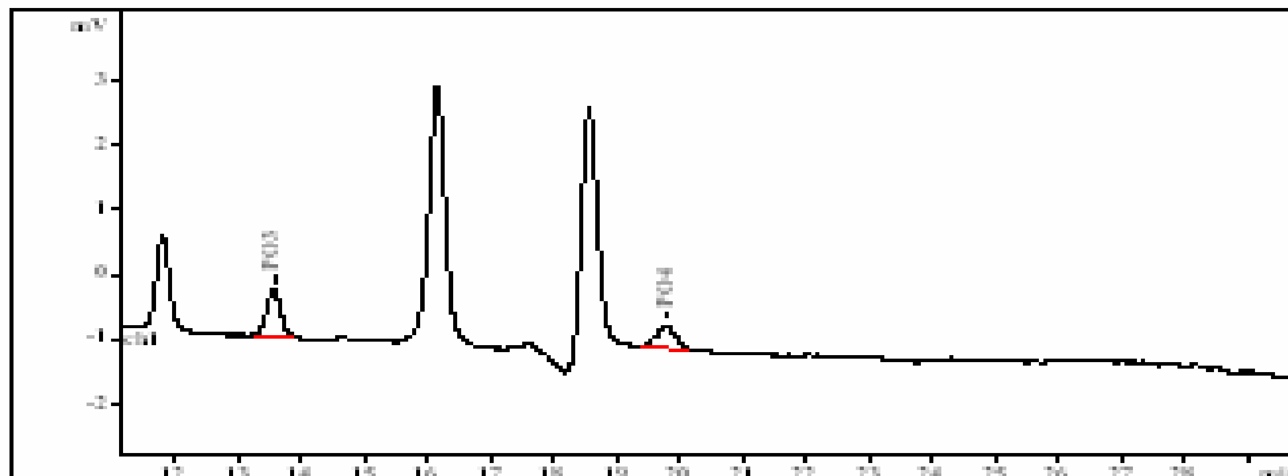

Fig 6: Chromatogram of LOD determination for phosphate (PO_4) and phosphite (PO_3)

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