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Determination and Control of Genotoxic Impurity in Vitamin E, Drug Substance

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ABSTRACT

Vitamin E (alpha-tocopherol) is a lipid-soluble antioxidant. p-toluenesulfonic acid, used as reagent in the synthesis of alpha-tocopherol, has a sulphonate group, a well-known alerting function for genotoxic activity. Genotoxic impurities in pharmaceuticals at lower levels are of increasing concerns not only to pharmaceutical industries but also for the regulatory agencies due to their risks for human carcinogenesis and, thus, requiring manufacturers to pay extra attention for their analysis and control. Therefore, sensitive and sophisticated analytical methodologies are deemed necessary in order to be able to test and control genotoxic impurities in drug substances. This work describes a simple, accurate and sensitive reversed-phase liquid chromatography—UV method for determination of trace amount of p-toluenesulfonic acid in pharmaceutical materials. A gradient mobile phase consists of acetonitrile and 0.01M potassium dihydrogen orthophosphate in water adjusted pH 3.0 with 0.1% phosphoric acid. Chromatography was carried out at ambient temperature 25°C on a Waters HPLC column C18, 150 mm x 4.6 mm, 5µm. The detection was carried out using variable wavelength UV-Vis detector set at 222 nm. The compounds were eluted at a steady flow rate of 1.0 mL/min. P-toluenesulfonic acid retention time was about 6.0 min with an asymmetry factor of 1.0. A logarithmic calibration curve was obtained from 0.1 to 3 µg/mL ($r > 0.9998$). Within-day %RSD was 0.21 ($n = 5$, 0.002 mg/mL) and between-day %RSD was 0.35 Specificity/ selectivity experiments revealed the absence of interference, recovery from spiked samples was between 97.6–103.78 percent. The developed method was applied to the determination of p-toluenesulfonic acid in pharmaceutical drug substance and drug product.

Keywords : P-toluenesulfonic acid; alpha-tocopherol; Reverse phase chromatography; Gradient elution. Vitamin E

I. INTRODUCTION

Starting materials, reagents, intermediates, byproducts, and degraded products are often found as impurities in active pharmaceutical ingredients (APIs). Some of these known impurities are mutagens or carcinogens with the potential to cause adverse effects on the human body, even at trace levels. Control and determination of these impurities at parts-per-million or parts-per-billion levels are significant challenges for analysts. When developing synthetic routes to APIs, it is the primary responsibility of laboratory personnel to identify the stages in which impurity generation can occur. The analyst must also identify and determine genotoxic impurities (GTIs) and control them at the stages of formation. The toxicologist must perform safety evaluations of high-priority compounds, known API impurities, and impurities with a high probability of occurring, and subsequently classify these compounds as genotoxic or routine impurities and propose limits. Various chromatography and spectroscopy methods help identify GTIs in an API. To detect and quantify the required levels and determine the signal-to-noise ratio, a derivatization must be performed, provided interference does not occur.

Guidelines from the International Conference on Harmonization (ICH) and EMA provide the limits for impurities in drug substances and drug products [1,2,3]. These limits do not apply to GTIs because of their adverse effects; hence it is necessary to determine limits based on the daily dose of the drug substance. This task drains process-development resources. To overcome this problem, scientists have to identify GTIs early in process development, develop analytical methods (i.e.,

for quantifying the genotoxic impurity), and demonstrate the necessary synthetic process controls.

EMA guidelines classify GTIs into two categories. The first, GTIs with sufficient experimental evidence for a threshold-related mechanism, is regulated using methods outlined in ICH Q3C (R4) for Class 2 solvents [4]. For the second category, GTIs without sufficient experimental evidence for a threshold-related mechanism, EMA proposes a "threshold of toxicological concern (TTC)" [5,6]. A TTC value of 1.5 g/day intake of a GTI is considered to be associated with an acceptable risk [7]. The concentration limit in ppm of GTI permitted in a drug substance is the ratio of TTC in μg/day and daily dose in g/day [8,9].

Driven by the FDA and EMEA guidelines, the pharmaceutical industry has developed several "compound-specific" analytical methods to determine known or suspected GIs in a pharmaceutical intermediate or drug substance [10]. A wide range of methods are used, including GC, GC–MS, LC, LC–MS and NMR. The development of these methods is often time- and resource-consuming.

P-toluenesulfonic acid is an aminoglycoside antibiotic used to treat various types of bacterial infections, particularly Gram-negative infections [11, 12]. It works by binding to a site on the bacterial 30S and 50S ribosome, preventing formation of the 70S complex. As a result, mRNA cannot be translated into protein and cell death ensues. P-toluene sulfonicacid is preferred over gentamicin for Pseudomonas aeruginosa pneumonia due to better lung penetration and bactericidal activity [13, 14].

United states Pharmacopoeia 2017 (USP 40) have described the procedure for assay of vitamin E by Gas chromatographic method but there is no any method of analysis reported for p-toluene sulfonicacid content determination by high performance liquid chromatography [15].

A simple reverse phase chromatographic methods have the advantages of short analysis time, enhancing sensitivity, flexibility and lowering the cost of the instruments and maintenance.

The literature survey shows that there are several methods like HPLC with UV-Visible detection or evaporative light scattering detection, or electrochemical detection, or fluorescence detection, LC/MS, GCMS methods have been reported for the determination of p-toluenesulfonic acid [16, 17]. But no any such kind of analytical method is available in literature for p-toulenesulfonic acid in Vitamin E drug substance.

The present study was aimed at developing simple, specific, accurate and precise HPLC method for determination of p-toluenesulfonic acid, genotoxic impurity in commercially available drug substance based on direct uv detection, in which gradient mobile phase based composition were used to p-toluenesulfonic acid, genotoxic impurity content in Vitamin E compounds, genotoxic impurity [18,19].

The proposed method for the determination of p-toluenesulfonic acid in pharmaceutical product by HPLC UV detectors is first of its kind without involving sophisticated instrument such as GCMS, LCMS and NMR. This information could be very useful for many of the pharmaceutical industries for the determination of p-toluenesulfonic acid compound and for those who do not have the costly instruments [20,21].

II. Experimental

2.1. Instrumentation

Modular high performance liquid chromatographic systems from Shimadzu Corporation (Chromatographic and Spectrophotometric Division, Kyoto, Japan) consisted of a tertiary gradient system, LC-20AT pump, SIL-10Advp autosampler, SPD-19A Advp UV-VIS detector, CTO-Acvp column oven, auto sampler and UV-Vis detector. HPLC column C-18, 150 mm x 4.6 mm, 5μm analytical column from Waters, was used as stationary phase. Chromatograms were recorded and integrated on PC installed with Class VP chromatographic software, version 5.032 (Shimadzu, Kyoto, Japan).

2.2. Reference substances, reagents and chemicals

Reference standard of p-toluenesulfonic acid was obtained from Sigma Aldrich, product of Japan. Potassium dihydrogen phosphate, orthophosphoric acid was purchased from Merck India. HPLC grade water was obtained from a Milli-Q system, Millipore, Milford,

MA, USA. All the chemicals and reagents were of analytical or reagent grade. Vitamin E drug substance containing p-toluenesulfonic acid were developed and manufactured in our research and development laboratory.

2.3. Chromatographic conditions

Gradient mobile phase consisted of a 0.01 M potassium dihydrogen phosphate, pH adjusted to 3.0 ± 0.05 using 0.1% orthophosphoric acid in water as mobile phase A and acetonitrile as mobile phase B. The gradient profile was Time - %B, 0-5min 10%, 15-18min 80%, 18.5-24min 10%. The Mobile phase was filtered and degassed through membrane filter of 0.45 µm porosity under vacuum. HPLC column C-18, 150 mm x 4.6 mm, 5µm analytical column was used as stationary phase. A constant flow rate of l.0 mL/min was employed throughout the analysis. Variable UV-Vis detector was set at 222 nm. All pertinent analyses were made at 25°C and volume of solution injected on to the column was 10 µl, mobile phase A was used as diluent for standard and sample preparations.

2.4. Samples

Test samples were Vitamin E drug substance synthesized in Research and development in-house laboratory.

2.5. Solution preparation

2.5.1. P-toluene sulfonic acid standard solution

P-toluene sulfonic acid standard solutions were prepared by transferring accurately about 101.5 mg of p-toluene sulfonic acid reference standard of anhydrous p-toluene sulfonic acid to a 100 mL volumetric flask. About 70 mL of diluent is added and sonicated for few minutes to solubilize p-toluene sulfonic acid. The solution was diluted to volume with the diluent and mixed. From this solution transferred 2mL in to 50mL volumetric flask and diluted with diluent and mixed. The solution was filtered through 0.45 µm membrane filter and 10 µL was injected.

2. 6. Estimation from drug substance

Five test preparations of drug substance were prepared of 1.0 mg/mL concentration and mixed. About 25.0mg of test sample was weighed accurately into a 25 ml volumetric flask, about 10 ml of diluent was added, shaken to dissolve the sample and diluted to volume with diluent and mixed. The solution was filtered through 0.45µm membrane filter and 10µl was injected directly on to the column.

2.7. Quantitation

Peak areas were recorded for all peaks. Peak areas were taken into account to quantitate the p-toluene sulfonic acid content in drug substance by using the following formula:

$$\text{P-toluene sulfonic acid mg/mL} = R_u / R_s \times C/100 \times 25/W \times P$$

Where R_u is peak area obtained from p-toluene sulfonic acid in the investigation solution; R_s is the peak areas obtained from p-toluene sulfonic acid in the standard solution; C is the weight, in mg, of p-toluene sulfonic acid working standard taken to prepare standard solution; W is the weight, in mg, of the test sample; P is purity of p-toluene sulfonic acid working standard.

III. RESULTS AND DISCUSSION

3.1. Chromatography

Our method development started with the search for the suitable column and mobile phase.

Chromatographic system comprising 0.02 M formic acid : acetonitrile (50 : 50 v/v), as mobile phase at a constant flow rate of l.0mL/min, Silica column, 250 mm x 4.0 mm, 5µm analytical column as stationary phase and detector wavelength at 220nm resulted in no peak elution even after 60 minutes of run time. Mobile phase consisting of 0.02 M aqueous potassium ammonium phosphate buffer and acetonitrile in the ratio 50:50, v/v, were tried in isocratic conditions on the Spherisorb ODS-1, 250 mm x 4.6 mm, 5µm to obtain symmetrical peak shapes, clear separation of the signal peaks from the solvent front peaks.

Up on investigation of following two chromatographic system containing 0.01 M potassium dihydrogen phosphate, pH adjusted to 7.0 using potassium hydroxide and 0.1M potassium dihydrogen phosphate,

pH adjusted to 6.9 using potassium hydroxide solution as mobile phase at a constant flow rate of l.0mL/min, Phenomenex C-18, 300 mm x 3.9mm,10µm and Purosphere RP-8e, 250 mm x 4.6 mm, 5µm analytical column as stationary phase and detector wavelength at 220nm resulted in peak elution at about 3.5 minutes which is very close to negative peaks of blank diluent. First investigation resulted in p-toluenesulfonic acid peak eluted very close to negative peak (peak from diluent) as shown figure 1.

Further, in order to develop a suitable and robust LC method for the determination of p-toluenesulfonic acid by UV detection different mobile phases and columns were employed (table 1) to achieve the best signal response and retention time.

Finally, the mobile phase consisting of water: 0.01 M potassium dihydrogen phosphate, pH adjusted to 3.0 ± 0.05 using orthophosphoric acid (0.1% solution in water) at a constant flow rate of 1.0 mL/min and detector wavelength set at 222 nm, using a Waters C-18, 150 mm x 4.6 mm, 5µm column was found to be appropriate, allowing good signal response of p-toluenesulfonic acid.

3.2 Optimization of HPLC

The pH of the mobile phase can affect the analyte's retention time as well as the detection sensitivity. Figure 2 shows the result of no peak elution of p-toluenesulfonic acid due to higher pH about 10. The optimal pH 3.0 ± 0.05 was chosen for the determination of p-toluenesulfonic acid elution with appropriate retention time.

The detection response is optimal at retention time at about 6 minutes at pH 3.0 of 0.01M potassium, dihydrogen phosphate with 0.1% orthophosphoric acid. Therefore, pH 3.0 and 0.01 M potassium dihydrogen phosphate was chosen for estimation of p-toluenesulfonic acid, typical chromatogram of test solution is shown in Figure. 3.

3.3. Method validation

Test method for the determination of p-toluenesulfonic acid was validated to include the essential demands of International conference on Harmonization (ICH) guidelines. Parameters like specificity, linearity, accuracy, precision, range and system suitability were examined.

3.3.1. Specificity

No interferences were observed due to obvious presence of mobile phase.

3.3.2. Linearity

Peak areas versus concentration in milligram per milliliter were plotted for p-toluenesulfonic acid at the concentration range between LOQ level to150 percent of target level. p-toluenesulfonic acid showed linearity in the range of 0.10-3.0 µg/mL with a correlation coefficient (r2) of 0.9998.

3.3.3. Accuracy

Accuracy of the proposed HPLC determination was evaluated from the p-toluene sulfonic acid content determination results of the components. Accuracy was done by performing the samples and calculated the peak area responses of different samples by component recovery method.

Stock solution

Stock Solution was prepared by dissolving accurately weighed about 101.5mg of p-toluene sulfonic acid in portions mobile phase and diluted to produce 100 mL solution.

Appropriate portions of stock solution were spiked into blank diluent to produce concentrations of 5, 12.5, 25, 50, 100 and 150 of target level. Mean recovery of spiked samples was 99.65 % for p-toluenesulfonic acid (Table 2).

3.3.4. Precision

Instrumental precision was determined by five replicate determinations of standard solution and the relative standard deviation was 0.21 for p-toluenesulfonic acid.
Method precision or intra-precision was performed by preparing six different samples involving different weighing. Each solution was injected in under same conditions and peak area responses for each solution were taken. Corrections in area were made for each weight taken to prepare six sample solutions. The

contents of P-toluenesulfonic acid in all six sample solutions were calculated. The p-toluenesulfonic acid content in all six preparations was found nil.

Intermediate precision was performed by analyzing the samples by two different instruments. Standard solution and six different samples at 100 percent target level were prepared. The p-toluenesulfonic acid content in all 12 preparations was found nil.

3.3.5. System suitability

System suitability tests were performed to chromatograms obtained from standard solutions to check the relative standard deviation of five replicate injections. Results obtained from five replicate injections of standard solution observed relative standard deviation less than 2.

3.4. Application of the proposed method

In-house prepared samples were evaluated for content of p-toluenesulfonic acid. The method gave reproducible results for all the samples tested for p-toluenesulfonic acid. The probable degradation products of test sample and p-toluenesulfonic acid did not interfere with the estimation of the component. The p-toluene sulfonic acid content, of the test samples are summarized in Table 3.

IV. Conclusion

A sensitive HPLC method based on UV detection has been developed and validated for determination of p-toluenesulfonic acid (genotoxic impurity) in Vitamin E drug substance. The method is simple, rapid, specific, accurate (error ±3.78%), precise (RSD <2.0%) and linear (r2=0.9998). The described method is suitable for routine analysis and quality control for determination of p-toluenesulfonic acid content in Vitamin E drug substance and drug product.

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Table 1. Employed mobile phases, columns and elution time during the investigation of p-toluenesulfonic acid

Column	Mobile phase	Elution time
Silica column, 250 mm x 4.0 mm, 5µm	0.02 M formic acid : acetonitrile (50 : 50 v/v)	*No peak eluted
Spherisorb ODS-1, 250 mm x 4.6 mm, 5µm	0.02 M ammonium phosphate : acetonitrile, (50 : 50 v/v)	*No peak eluted
Phenomenex C-18, 300 mm x 3.9 mm, 10µm.	0.01 M potassium dihydrogen phosphate, pH adjusted to 7.0 using Potassium hydroxide.	**3.5 minutes
Purosphere RP-8e, 250 mm x 4.6 mm, 5µm	0.1M potassium dihydrogen phosphate, pH adjusted to 6.9 using potassium hydroxide solution	**3.2 minutes
Waters, Spherisorb ODS-1, 250 mm x 4.6 mm, 5µm	0.05 M aqueous diammonium hydrogen, pH 10 with tetramethyl ammonium hydroxide : acetonitrile, (50 : 50 v/v)	*No peak eluted
Waters C-18, 150 x 4.6 mm, 5µm.	0.01 M potassium dihydrogen phosphate, pH adjusted to 3.0 using 0.1% orthophosphoric acid	About 6.0 minutes.

*****No peak p-toluenesulfonic acid eluted after 60 minutes time

****** P-toluenesulfonic acid peak eluted very close to negative peak (peak from diluents)

Table 2. Accuracy data (analyte recovery): P-toluenesulfonic acid

	Amount std. added(µg/mL)	Amount std. recovered (µg/mL)	Determined (% of target level)	Recovered (%)	Bias (%)
1	0.1015*	0.103	5.07	98.54	-1.46
2	1.015*	1.030	50.73	98.16	-1.84
3	2.030*	2.080	102.46	97.60	-2.40
4	3.045*	2.930	144.33	103.78	3.78

*Average of three determination

Table 3 : Application of the developed HPLC method for the determination of p-toluenesulfonic acid in drug substance and drug product

Sr. No.	Test sample	PTSA Content found to be
1	Drug substance	Nil
2	Evion tablets	Nil
3	Biotrex vitamin E capsules	Nil

Figures and captions

Fig 1.

Chromatogram of p-toluenesulfonic acid showing first investigated chromatographic column: Phenomenex C-18, 300 mm x 3.9mm, 10µm, mobile phase: 0.01 M potassium dihydrogen phosphate, pH adjusted to 7.0 using potassium hydroxide, flow rate 1ml/min. Detector wavelength: 220 nm

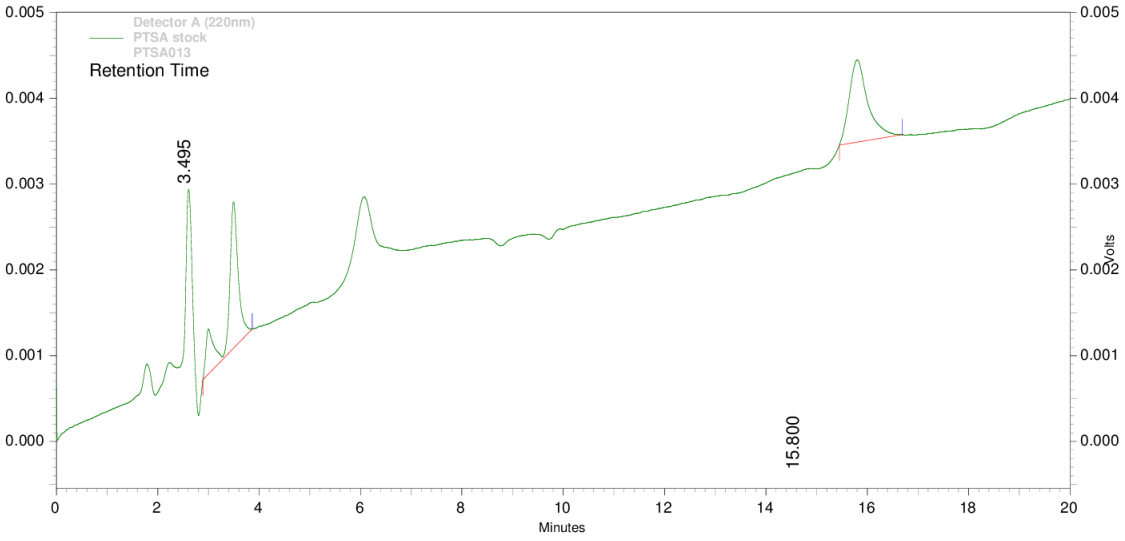


Fig 2. Effect of pH on peak elution of p-toluene sulfonic acid column: Purosphere RP-8e, 250 x 4.6 mm, 5μm, mobile phase: 0.05 M diammonium hydrogen phosphate, pH adjusted to 10 using tetramethyl ammonium hydroxide

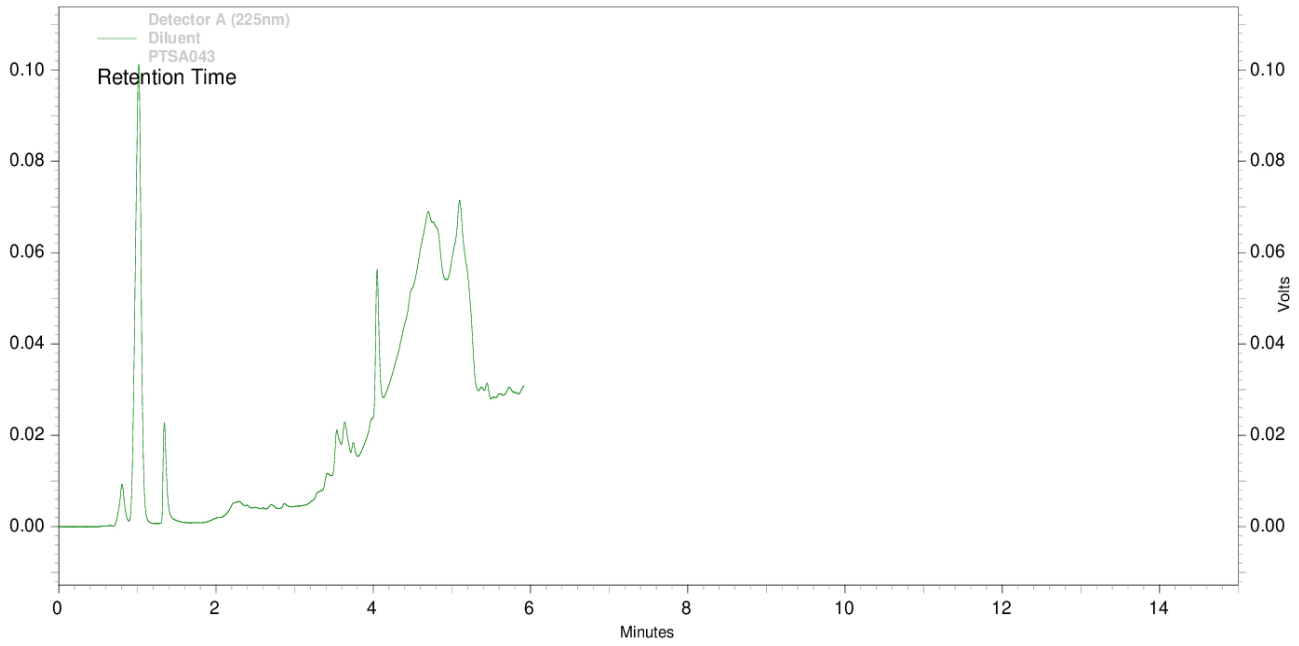
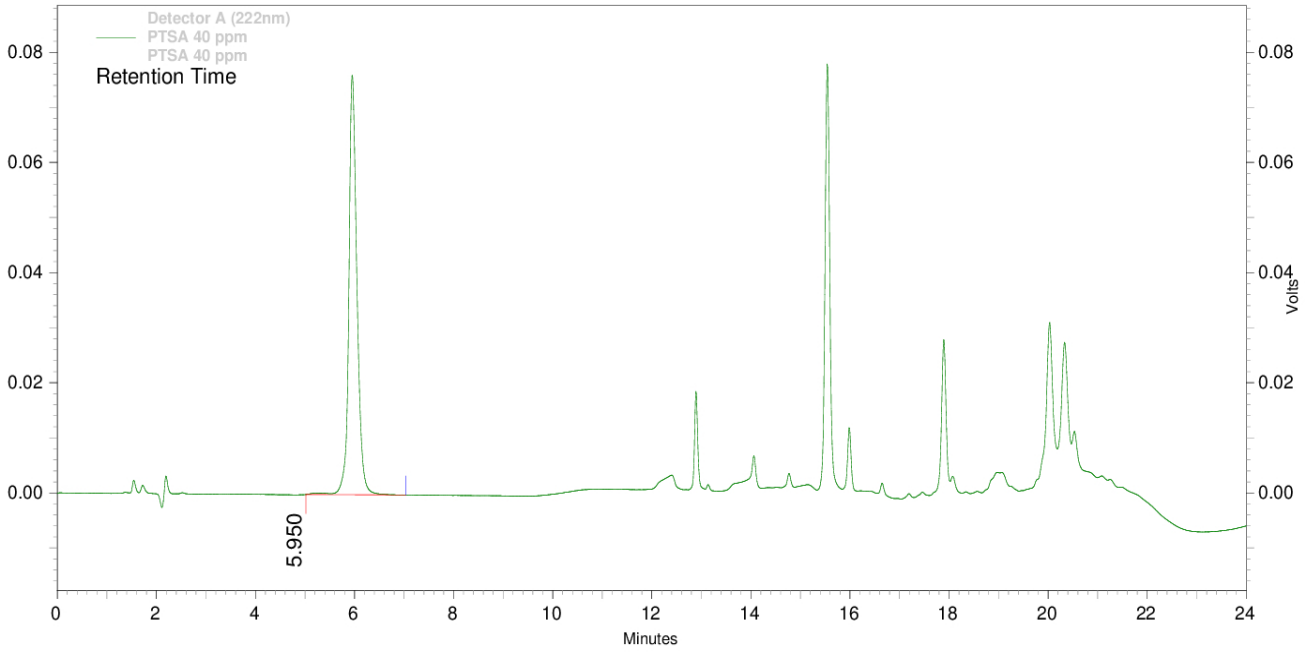


Fig 3. A typical chromatogram of test sample by proposed methods column: Waters C-18, 150 x 4.6 mm, 5μm, gradient mobile phase: A-0.01 M potassium dihydrogen phosphate and B-acetonitrile, pH adjusted to 3.0 using 0.1% orthophosphoric acid, flow rate 1ml/min, λ set at 222 nm.



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