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Article

Stability-indicating RP-HPLC method for simultaneous estimation of levosalbutamol sulfate and theophylline in combined dosage form

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A novel, simple, accurate and precise RP-HPLC method for simultaneous determination of levosalbutamol sulfate and theophylline has been developed and validated. Separation was achieved on a Phenomenex; C_{18} column (250 mm × 4.6 mm i.d., 5 µm) using methanol: 10 mM TBAHS(tetrabutyl ammonium hydrogen sulfate) (50:50, v/v) as mobile phase at flow rate of 1.0 $mL.min^{-1}$. The UV detection wavelength was 274 nm. The linearity is obeyed over a concentration range of 0.5-150 $\mu g.mL^{-1}$ with correlation coefficient of 0.999 for both the drugs. The proposed method was validated by determining accuracy, precision, stability and system suitability parameters. The method was found to be robust. Specificity of the method was determined by subjecting the drugs to various stress conditions like acid, alkali, oxidation, thermal and photolytic degradation. The method was used successfully for the simultaneous determination of levosalbutamol sulfate and theophylline in syrup dosage form.

Uniterms: Levosalbutamol/determination. Theophylline/determination. Stability-indicating. RP-High performance liquid chromatography/qualitative analysis Syrup/qualitative analysis.

Desenvolveu-se e validou-se método de RP-HPLC novo, simples, exato e preciso de determinação simultânea do sulfato de levossalbutamol e teofilina.. A separação foi efetuada em uma coluna Phenomenex; C18 (250 mm x 4,6 mm d.i., 5 µm) utilizando metanol: TBAHS (hidrogenossulfato de tetrabutilamônio) 10 mM (50:50, v/v) como fase móvel, com fluxo de 1,0 $mL.min^{-1}$. O comprimento de onda de detecção no UV foi 274 nm. Observou-se linearidade na faixa de concentração de 0,5-150 $\mu g\ mL^{-1}$, com coeficiente de correlação de 0,999 para ambos os fármacos. O método proposto foi validado determinando-se exatidão, precisão, estabilidade e parâmetros de adequação do sistema. O método mostrou-se robusto. A especificidade do método foi determinada submetendo os fármacos a várias condições de estresse, como ácido, álcali, oxidação, degradação térmica e fotolítica. O método foi usado com sucesso para a determinação simultânea do sulfato de levossalbutamol e teofilina na forma de xarope.

Unitermos: Levosalbutamol/determinação. Teofilina/determinação. Indicador de estabilidade. RP-Cromatografia líquida de alto desempenho. Xarope/análise qualitativa

INTRODUCTION

Levosalbutamol sulfate (levalbuterol), (*R*)-α´-[(*tert*-butylamino)-4-hydroxy-*m*-xylene-αα´-diol sulfate(2:1) is a bronchodilator indicated for management of asthma. It is preferred over racemic salbutamol sulfate for increased efficacy and safety after use (Berger, 2003). Theophylline, 3,7-dihydro-1,3-dimethyl purine-2,6(1*H*)-dione is a xanthine derivative used for management of asthma and related obstructive respiratory conditions(Persson,1986). The chemical structure of both the drugs are shown in Figure 1.

Literature review shows that no stability-indicating RP-HPLC methods are present so far for simultaneous estimation of levosalbutamol sulfate and theophylline in combined syrup dosage form using methanol: 10 mM TBAHS (tetra butyl ammonium hydrogen sulfate) as the mobile phase. Some of the reported methods for racemic salbutamol sulfate includes titrimetric (Basavaiah *et al*., 2006, 2007), potentiometric (Abdel-Ghani *et al*., 2002), spectrophotometric (Basavaiah *et al*., 2003, 2006, 2007; Chitlange *et al*., 2011; Culzoni *et al*., 2006; Geeta *et*

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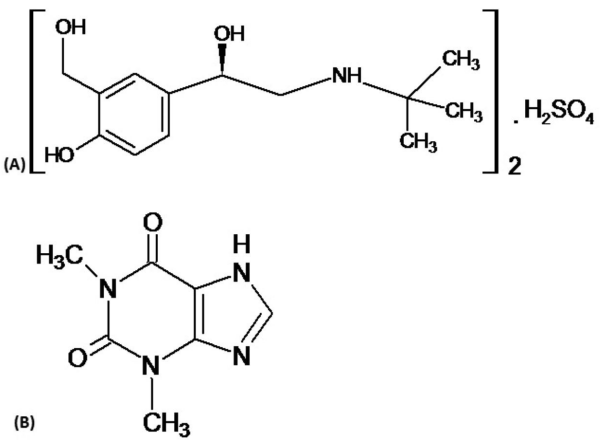

FIGURE 1 - Chemical Structure of (A) levosalbutamol sulfate and (B) theophylline.

al., 1989; Habib *et al*., 2004; Jain *et al*., 2011; Mishra *et al*., 2010; Parimoo *et al*., 1993; Rathore *et al*., 1993), spectrofluorimetric (Pandya *et al*., 2010), HPLC (Bernal *et al*., 1996; Boulton *et al*., 1995; Chitlange *et al*., 2011; Erram *et al*., 2006; Jain *et al*., 2011; Kasawar *et al*., 2010; Kountourellis *et al*., 1990; Martis *et al*., 2011; Murtaza *et al*., 2009; Pai *et al*., 2009) and LC-MS(Joyce *et al*., 1998; Wu *et al*., 2004, 2011; Yee *et al*., 2011). Whereas for levosalbutamol sulfate a few spectrophotometric (Prasanna *et al*., 2012; Thulasama *et al*., 2009) and HPLC (Ghemud *et al*., 2012; Halabi *et al*., 2004; Prasanna *et al*., 2012) methods are reported. The reported methods for theophylline includes spectrophotometric (Abuirjeie *et al*., 1992; Bermejo *et al*., 1985; Carter *et al*., 1985; Fellenberg *et al*., 1979; Hohnadel *et al*., 1978; Jatlow, 1975; Kumar *et al*., 2011; Plavsic, 1978; Vasiliades *et al*., 1976), HPLC (Abuirjeie *et al*., 1992; Boberic-Borojevic *et al*., 1999; Greenberg *et al*., 1979; Lauff, 1987; Moncrieff, 1991; Papadoyannis *et al*., 1993; Shidhaye *et al*., 2009), GC (Chrzanowski *et al*., 1974; Schwertner, 1979; Shah *et al*., 1974) and LC-MS (Abdel-Hamid *et al*., 2003; Babu *et al*., 2011; Kanazawa *et al*., 2000; Song *et al*., 2004). The literature survey suggests there is lack of a stability indicating RP-HPLC method for simultaneous determination of levosalbutamol sulfate and theophylline in combined syrup dosage forms. Only one RP-HPLC method has been reported so far for simultaneous estimation of the racemic salbutamol sulfate and theophylline in combined tablet dosage form (Maithani *et al*., 2011). But the reported method has several drawbacks like maintaining pH of buffer, increased retention times of the drugs, narrow range of linearity and higher mobile phase flow rate etc. So, an attempt was made to overcome these drawbacks and develop a novel, simple, accurate and precise, stability-indicating RP-HPLC method for simultaneous determination of levosalbutamol sulfate and theophylline in syrup dosage form. To prove the stability-indicating nature of the method forced degradation of drug substances and drug product was performed under stress conditions (acid, alkali, oxidation, thermal and photolysis), and the stressed samples were analyzed by the developed method. The method was also validated according to requirements of ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2005) guideline.

MATERIAL AND METHODS

Material

Methanol used was of HPLC grade (Merck Ltd., Mumbai, India).Water for chromatography was obtained using TKA water purification system (Germany). Standard drug of levosalbutamol sulfate (purity>99.5%) and theophylline (purity>99%) were received from Cipla Ltd., India and Glenmark Pharmaceuticals Ltd., India, respectively. AR grade Tetra Butyl Ammonium Hydrogen Sulfate (TBAHS) was procured from HiMedia Laboratories Pvt. Ltd., India. Marketed syrup formulation was bought from the local market.

Chromatographic conditions

Quantitative HPLC was performed on a binary gradient HPLC with Shimadzu LC-10AT and LC-10AT VP Series HPLC pumps, with a 20 µL sample injection loop (manual) and SPD 10A VP series UV-Visible detector. The signal was monitored and integrated using Shimadzu Class-VP Version 6.12 SP1 Software. A Phenomenex; C_{18} column (250 mm× 4.6 mm i.d., 5 µm) was used for separation. Methanol: 10 mM TBAHS in the ratio (50:50, v/v) was used as the mobile phase. The 10 mM TBAHS solution was prepared by accurately weighing 3.3954 g of TBAHS salt and dissolving it in 1000 mL of HPLC grade water. Afterwards, both the methanol and TBAHS solution were ultrasonicated up to 30 min for degassing and were filtered through a 0.45 µm membrane filter, prior to use. The flow rate was 1.0 $mL.min^{-1}$ and UV detection was carried out at 274 nm. The separation was achieved at room temperature.

Preparation of standard stock solution

Standard stock solutions for both the drugs were prepared separately by dissolving 25 mg of drugs in mobile phase up to 25 mL. The volumetric flasks having

10 mL of mobile phase with the drugs were shaken and finally volume was made up to get a concentration of 1000 µg.mL^{-1}. Both the standard drug solutions were filtered through a 0.2 µm membrane filter. The stock solutions were stored at 2-8 ºC.

Method validation

Linearity and preparation of analytical curve

Suitable aliquots of the standard stock solutions of levosalbutamol sulfate (1000 µg.mL^{-1}) and theophylline (1000 µg.mL^{-1}) were transferred into a series of 10 mL volumetric flasks to get final concentrations of 0.5, 1.0, 5.0, 10, 20, 40, 80,100,150 µg.mL^{-1}(9 points). Final volume was made up with mobile phase. Each mixed standard solution was injected and chromatograms were recorded. The linearity of analytical curves was plotted for peak area of each drug against concentrations of drug.

Analysis of syrup dosage form

Five mL of the syrup containing levosalbutamol sulfate and theophylline equivalent to 5mg and 50mg of the standard drugs respectively was measured accurately and dissolved in 50 mL of mobile phase. It was ultrasonicated for 25 minutes and volume was made up with the mobile phase, followed by filtration through 0.2 µm membrane filter. The syrup solution was further diluted with the mobile phase to get sample solutions within the linearity range. The sample solutions were injected and chromatograms were obtained. The amount of drug present in the sample solution was calculated using the analytical curves of standard drugs.

Precision

The precision (intraday and interday) of the method was ascertained from the peak areas obtained by determination of six replicates of fixed amount of drugs. The system precision was ascertained by carrying out six injections of a fixed amount of drug solution. The percent relative standard deviations were calculated.

Accuracy

To check the accuracy of the proposed method, recovery studies were carried out at 80, 100 and 120% of the test concentration according to ICH guidelines. The recovery study was performed three times at each level.

Robustness

Robustness of the method was studied by deliberately changing the UV detection wavelength (±5 nm), mobile phase flow rate (±0.2 mL) and organic phase composition (±5%).The effect was studied in terms of various system suitability parameters like retention time, resolution, theoretical plates and tailing factor for both the drugs. Solution stability study was also carried out by keeping the sample solutions at 25±2 ºC for 24 h.

Forced degradation study

Specificity of the method was determined by checking interference of any of the possible degradation products produced during the forced degradation study. The forced degradation was carried out with 0.1 M HCl, 0.1 M NaOH, 3% v/v hydrogen peroxide, thermal (80 ºC) and photolysis (365 nm) for finding out the stability nature of the drugs. The degradation samples were prepared by taking suitable aliquots of the drug and drug product solutions, and then undertaking the respective stress testing procedures for each solution. After the fixed time period the treated drug solutions were diluted with mobile phase. For every stress condition three solutions were prepared as 1 µg.mL^{-1} of levosalbutamol sulfate, 10 µg.mL^{-1} of theophylline and drug product solution containing 1 µg.mL^{-1} of levosalbutamol sulfate with 10 µg.mL^{-1} of theophylline. The specific stress conditions are described as follows.

A) Acidic degradation

Acidic degradation was carried out by adding 1.0 mL of 0.1 M HCl, and after 1hr neutralizing the mixture by adding 0.1 M NaOH.

B) Alkali degradation

Alkali degradation was carried out by adding 1.0 mL of 0.1 M NaOH, and after 1 h neutralizing the mixture by adding 0.1 M HCl.

C) Oxidative degradation

Oxidative degradation was performed by exposing the drug to 1.0 mL of 3% (v/v) H_2O_2 1 h.

D) Thermal degradation

Thermal degradation was performed by heating the drug solutions at 80 ºC on a thermostatically controlled water bath for 1 h.

E) Photolytic degradation condition

Photolytic degradation was carried out by exposing the drug solutions to UV light (365 nm) inside an UV chamber for 5 h.

System suitability

The System Suitability was calculated for different parameters like theoretical plates, resolution, tailing factor, LOD and LOQ. The LOD and LOQ were separately determined based on the Signal to Noise ratio. For LOD the S/N ratio is 3:1.For LOQ the S/N ratio is 10:1.

RESULTS AND DISCUSSION

Method development and optimization

Method development process was carried out by examining conditions like flow rate (0.8 mL.min^{-1}, 1.0 mL.min^{-1} and 1.2 mL.min^{-1}), mobile phase compositions like acetonitrile:water, acetonitrile:buffer, methanol:water, methanol: 10 mM TBAHS and ratios (50:50, 60:40 and 45:60, v/v) were used. Both the drugs levosalbutamol sulfate and theophylline were found showing a significant UV absorbance at 274 nm in methanol: 10 mM TBAHS (50:50, v/v), so this wavelength was chosen for UV detection. By use of a C_{18} column it was found the mobile phase consisting of methanol: 10mM TBAHS (50:50, v/v) provided well defined peak shape with good resolution. Levosalbutamol sulfate was eluted at 2.558 min where as theophylline eluted at 4.867 min. The representative chromatograms of pure drug and combined drug product are shown in Figure 2 (A) and (B), respectively.

Method validation

Linearity

Levosalbutamol sulfate and theophylline have linearity over concentrations ranging from 0.5 to 150.0 µg.mL^{-1}. The slope (a) and intercept (b) were found to be 6103 and 4583 for levosalbutamol sulfate and 63827 and -2793 for theophylline. Correlation coefficient (r^2) was found to be 0.999 for both the drugs. These results suggest a good linear relationship between peak area and analytes in the range studied.

Analysis of syrup dosage forms

The % recovery was more than 100% for syrup dosage form. Good % recovery and noninterference in separation of both the drugs because of formulation components suggests that; the method is selective and excipients present in the formulation have no affect in the determination. The results are given in Table I.

Precision

The RSD for method (intraday and interday) and system precision were found to be less than 2% showing high degree of preciseness as shown in Table II.

Accuracy

Accuracy was checked by preparing mixtures containing different amounts of pure drugs and fixed amount of formulation; analyzing the mixtures by use

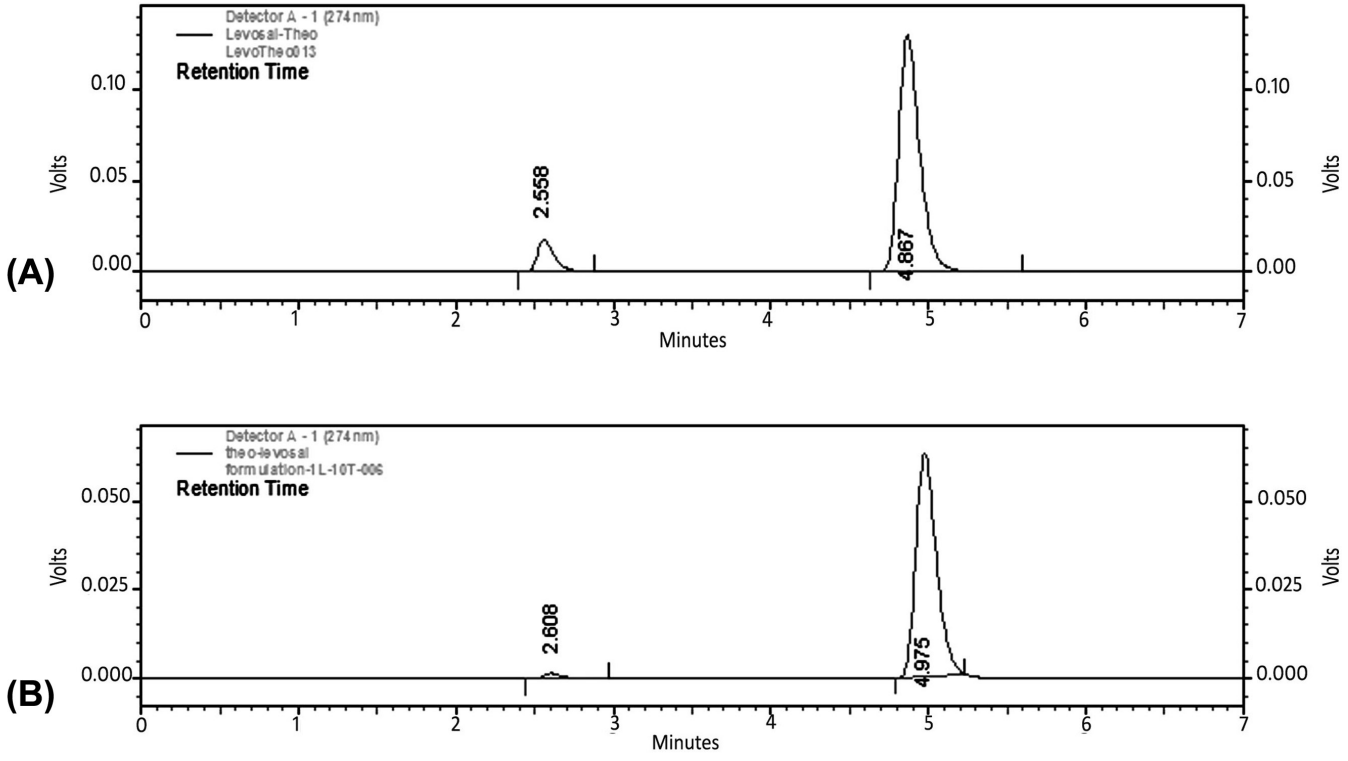

FIGURE 2 - Representative chromatograms of levosalbutamol sulfate and theophylline. (A) combined standard drugs[ratio 1:1] (B) combined formulation[ratio 1:10].

TABLE I - Analysis of syrup dosage form

Formulation Type	Labeled Amount (mg)	Recovery by proposed method[a] (%)±S.D., R.S.D (%)	
		Levosalbutamol sulfate	Theophylline
Syrup	Each 5 mL contains levosalbutamol sulfate≈5 mg theophylline≈50 mg	101.6±1.4106, 1.38	101.43±0.9237, 0.91

[a] average of three determinations

TABLE II - Intraday, interday and system precision studies(n=6)

Type of Study	Concentration Taken (μg.mL^{-1})		Peak Area[b] ±Standard Deviation,(%RSD)	
	Levosalbutamol Sulfate	Theophylline	Levosalbutamol sulfate	Theophylline
Intraday precision	20	20	125306.83± 260.13,0.21	1246288± 4391.99,0.35
Interday precision	20	20	126566± 356.59,0.28	1246723± 3592.9,0.29
System precision	20	20	126234.16± 366.88,0.29	1264023± 3545.68,0.28

[b] average of six determinations

of developed method. Percentage recovery and relative standard deviation were also calculated. The results obtained suggest the recoveries were excellent, and relative standard deviations were less than 2%.The results of recovery study are given in Table III.

Robustness

The method was found to be robust under deliberate changes made in mobile phase flow rate, composition of organic phase and UV detection wavelength. Various system suitability parameters were evaluated to find out the robustness of the method. The results for system suitability parameters are presented in Table IV. The results of solution stability for both the drugs was satisfactory with a recovery more than 99% after 24 h, showing no significant loss of the analytes in the selected mobile phase.

Specificity

Specificity of the method was ascertained by checking any interference because of excipients or degradation products produced after subjecting levosalbutamol sulfate and theophylline to forced degradation. No extra peaks were obtained either from the excipients used in the drug product or from the stress conditions applied on the drugs and drug product. Therefore the method was found specific and stability-indicating. Forced degradation conditions were optimized in order to get degradation of the drugs up to 30%. The stress conditions like 0.1 M HCl, 0.1 M NaOH, 3% v/v H_2O_2, 80 ºC temperature and 365 nm UV radiation were applied to check the degradation nature of the drugs. Levosalbutamol sulfate undergoes significant degradation (34.15%) in oxidative stress, moderate degradation in alkaline (5.64%) and thermal (3.67%)

TABLE III - Accuracy of the method

Different concentration level comparing to sample concentration (%)	Amount Added Pure Drug (μg.mL^{-1})		Recovery[c](%) Pure Drug ±SD, RSD (%)	
	Levosalbutamol Sulfate	Theophylline	Levosalbutamol Sulfate	Theophylline
80	4	40	100.5±0.4330,0.43	101.11±0.5478,0.54
100	5	50	100.43±0.1571,0.16	100.61±0.2402,0.24
120	6	60	100.33±0.33,0.33	100.81±0.4463,0.44

[c] average of three determinations at each level

TABLE IV- Robustness of the method

Parameter	RetentionTime(Rt),min		Resolution (Rs)	Theoretical Plates(N)		Tailing Factor(T)	
	Levosalbutamol sulfate	Theophylline		Levosalbutamol sulfate	Theophylline	Levosalbutamol sulfate	Theophylline
Flow rate ($mL.min^{-1}$)							
0.8	3.167	6.042	5.75	2938	5945	1.39	1.33
1.0	2.575	4.967	5.98	2926	5989	1.36	1.33
1.2	2.142	4.083	5.54	2838	5427	1.36	1.33
Methanol (%)							
45	2.592	5.433	6.31	2899	5685	1.31	1.39
50	2.575	4.967	5.98	2926	5989	1.36	1.33
55	2.533	4.483	5.57	2992	5673	1.31	1.36
Wavelength (nm)							
269	2.575	4.975	6.0	2941	5900	1.31	1.34
274	2.575	4.967	5.98	2969	5989	1.36	1.33
279	2.558	4.875	5.79	2895	5695	1.34	1.37

stress conditions. Levosalbutamol was found to be stable in the applied acidic and UV stress conditions. The drug theophylline was found to be more stable towards the applied stress conditions than compared to levosalbutamol. Theophylline undergoes moderate degradation in alkaline (7.57%) and oxidative (2.47%) stress conditions, and the drug was found to be stable in the applied acidic, thermal and UV stress conditions. Figure 3-(A), (B),(C),(D),(E) and (F) represents the chromatograms of untreated drugs; acid, alkali, oxidation, thermal and photolysis degraded drugs with combined drug product, respectively.

System suitability

A critical evaluation of the method was performed. A higher resolution value indicates better separation of both the drugs. Also the LOD and LOQ values show superior sensitivity of the method. The System suitability parameters are shown in Table V.

TABLE V- System suitability

Parameter	Obtained Values	
	Levosalbutamol sulfate	Theophylline
Retention time	2.558	4.867
Theoretical plates	2926	5989
Tailing factor	1.36	1.33
Resolution	---	5.94
LOD($\mu g.mL^{-1}$)	0.16	0.015
LOQ($\mu g.mL^{-1}$)	0.54	0.051

CONCLUSION

This simple and accurate RP-HPLC method has been developed and validated for determining levosalbutamol sulfate and theophylline in syrup dosage form. Lack of a stability-indicating method for determination of both the drugs in a combined syrup dosage form is the driving cause for development of this novel method. The sample preparation technique is also very simple for the syrup dosage form making it suitable for routine laboratory testing. Major advantage of this method is the analytes do not interfere with each other's elution with a good difference in retention times. Specificity study carried out by forced degradation ensures that the method is specific and able to describe the stability nature of both the drugs in a combined drug formulation. The mobile phase is simple to prepare. Results of precision study demonstrate the superior preciseness of the method as the RSD values were well within the limits. The recovery value of more than 100% shows higher levels accuracy of the method. So it can be concluded that the developed RP-HPLC method is novel, simple, accurate, precise, sensitive, and stability-indicating and can be employed successfully for the simultaneous determination of levosalbutamol sulfate and theophylline in syrup dosage form. Further the developed method may be applied for bioavailability and bioequivalence study of both the drugs in different biological samples.

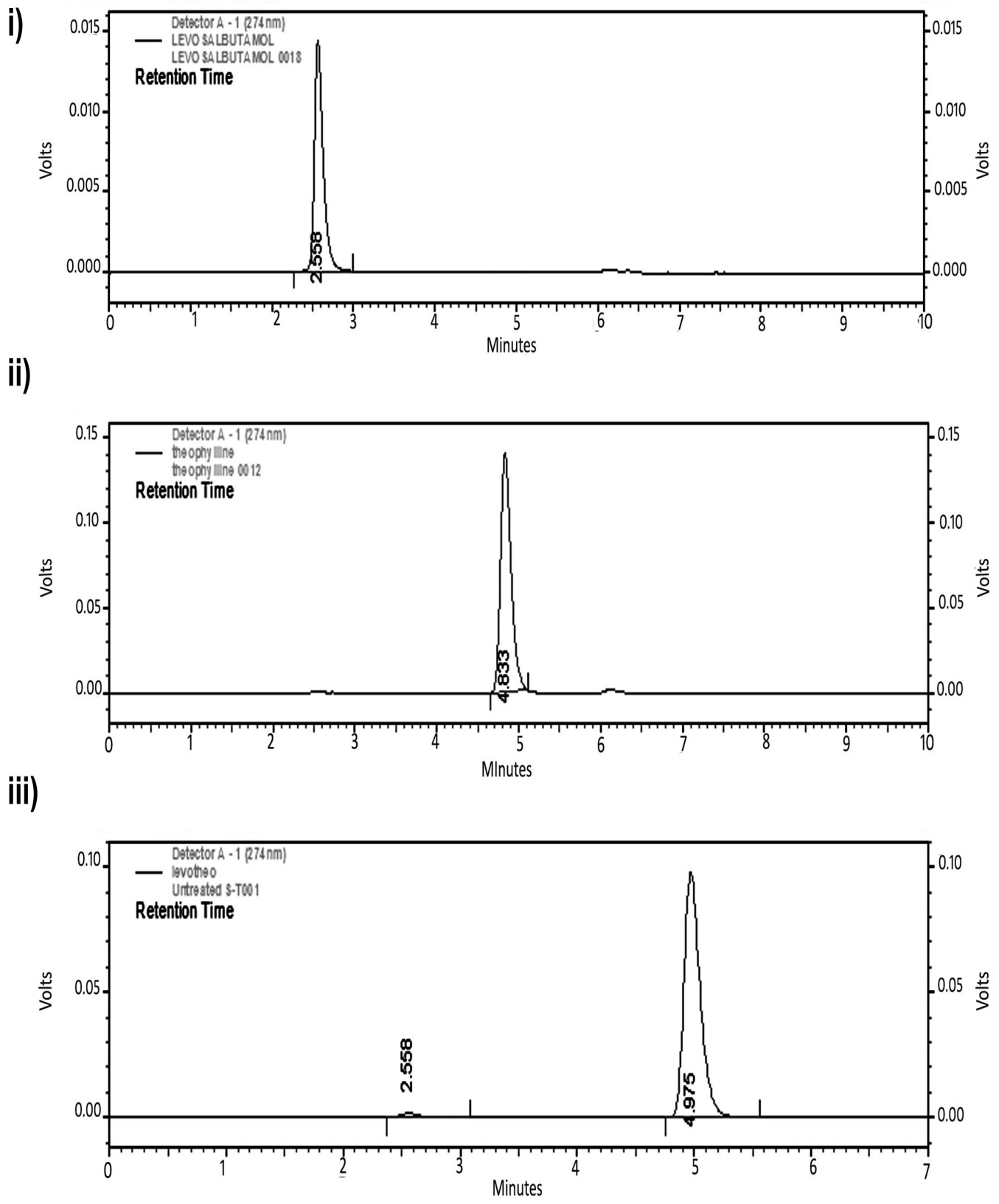

FIGURE 3A - Representative chromatograms of untreated drugs. Each chromatogram represents (i) levosalbutamol sulfate (1 μg.mL^{-1}), (ii) theophylline (10 μg.mL^{-1}), (iii) dosage form having levosalbutamol sulfate (1 μg.mL^{-1}) and theophylline (10 μg.mL^{-1}).

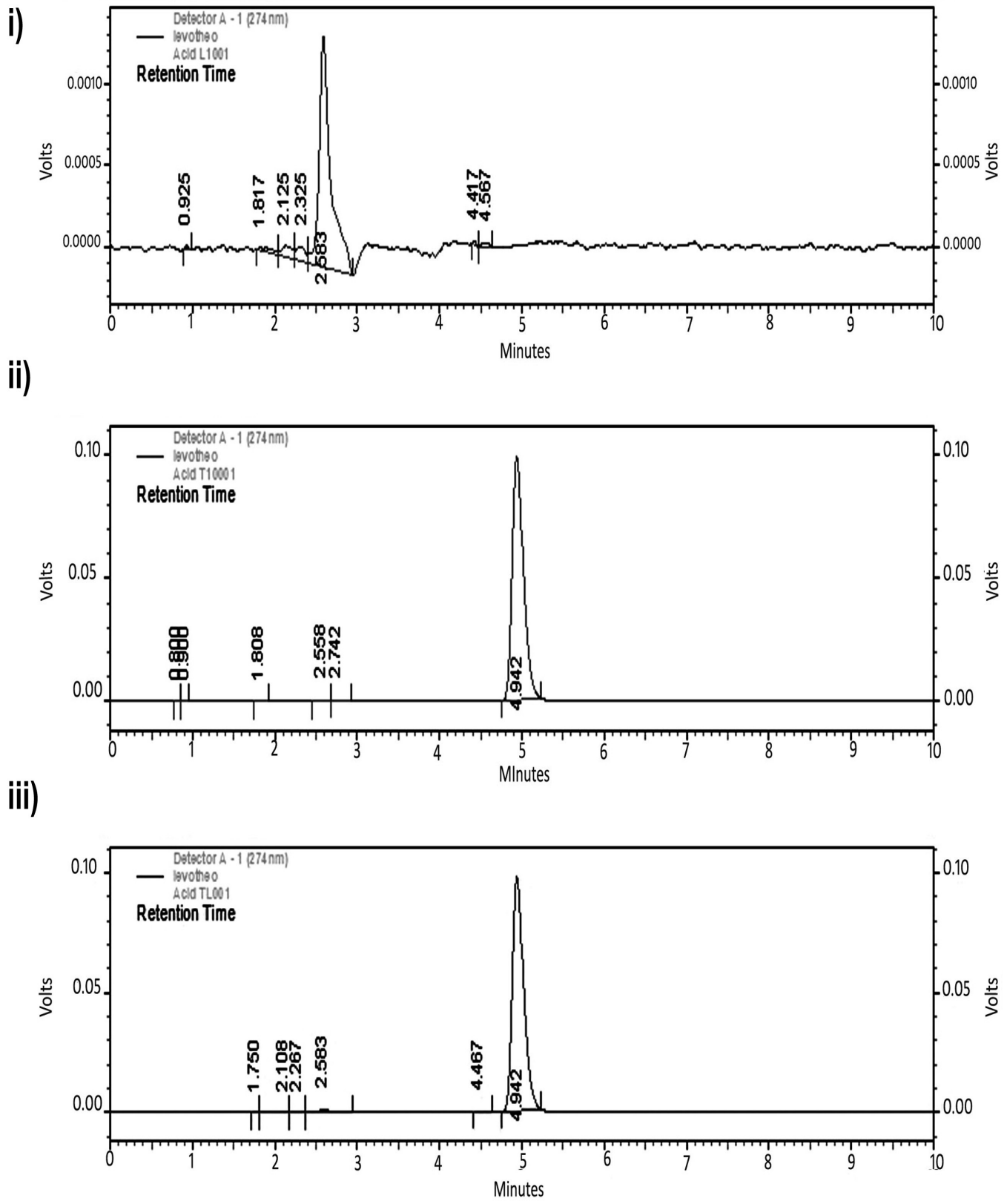

FIGURE 3B - Representative chromatograms of acid degraded drugs. Each chromatogram represents (i) levosalbutamol sulfate (1 μg.mL^{-1}), (ii) theophylline (10 μg.mL^{-1}), (iii) dosage form having levosalbutamol sulfate (1 μg.mL^{-1}) and theophylline (10 μg.mL^{-1}).

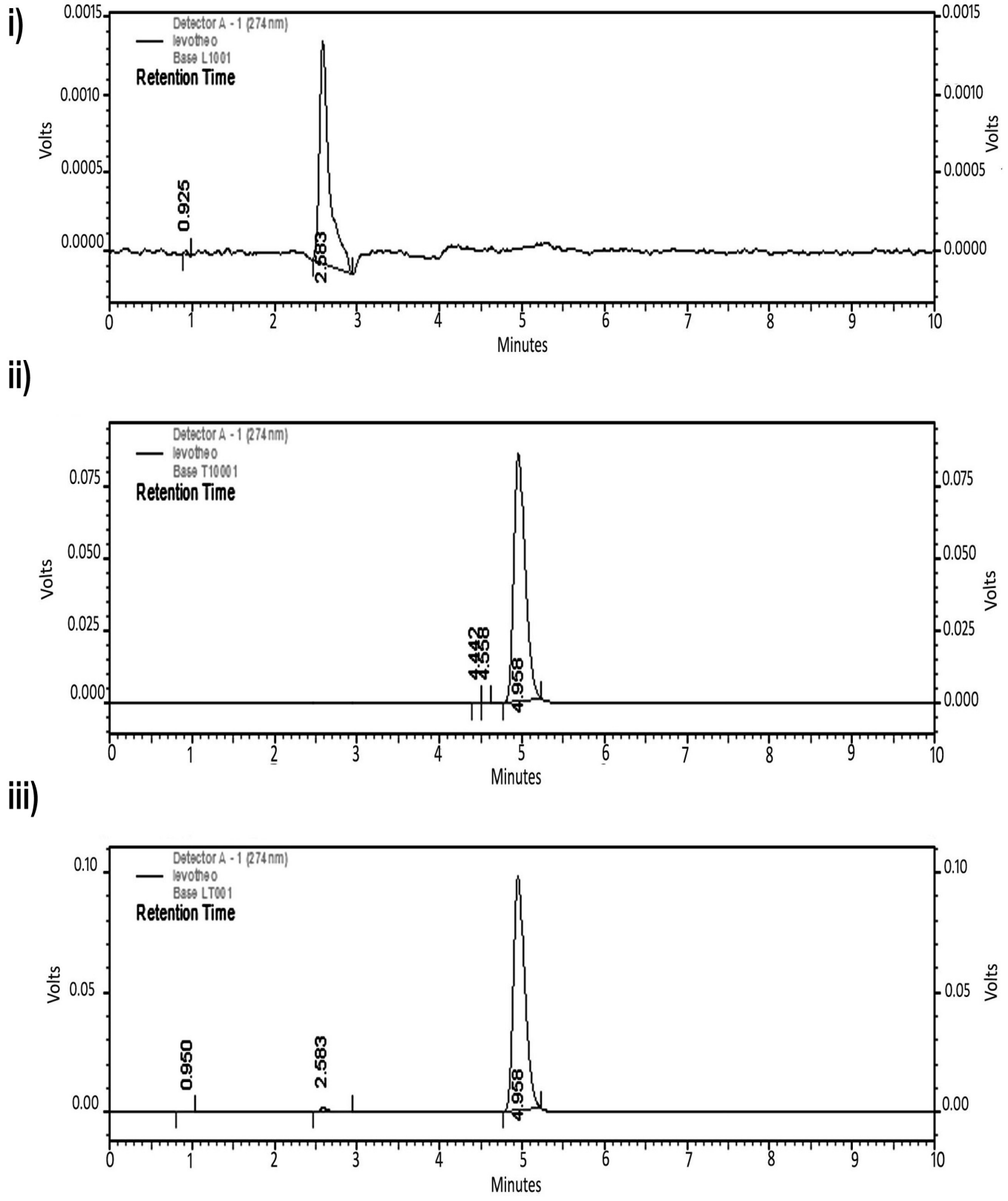

FIGURE 3C - Representative chromatograms of alkali degraded drugs. Each chromatogram represents (i) levosalbutamol sulfate (1 μg.mL^{-1}), (ii) theophylline (10 μg.mL^{-1}), (iii) dosage form having levosalbutamol sulfate (1 μg.mL^{-1}) and theophylline (10 μg.mL^{-1}).

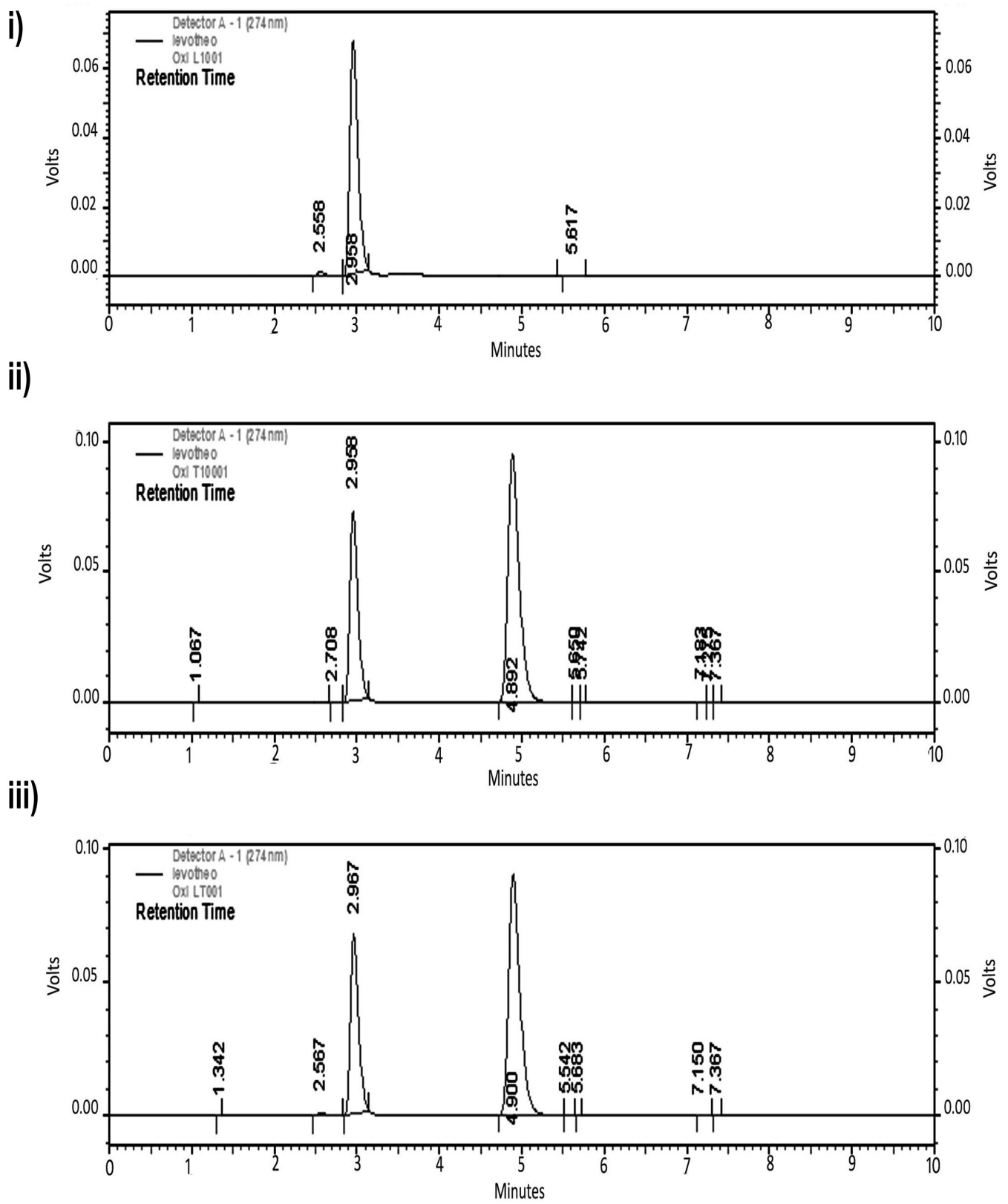

FIGURE 3D - Representative chromatograms of oxidation degraded drugs. Each chromatogram represents (i) levosalbutamol sulfate (1 μg.mL^{-1}), (ii) theophylline (10 μg.mL^{-1}), (iii) dosage form having levosalbutamol sulfate (1 μg.mL^{-1}) and theophylline (10 μg.mL^{-1}).

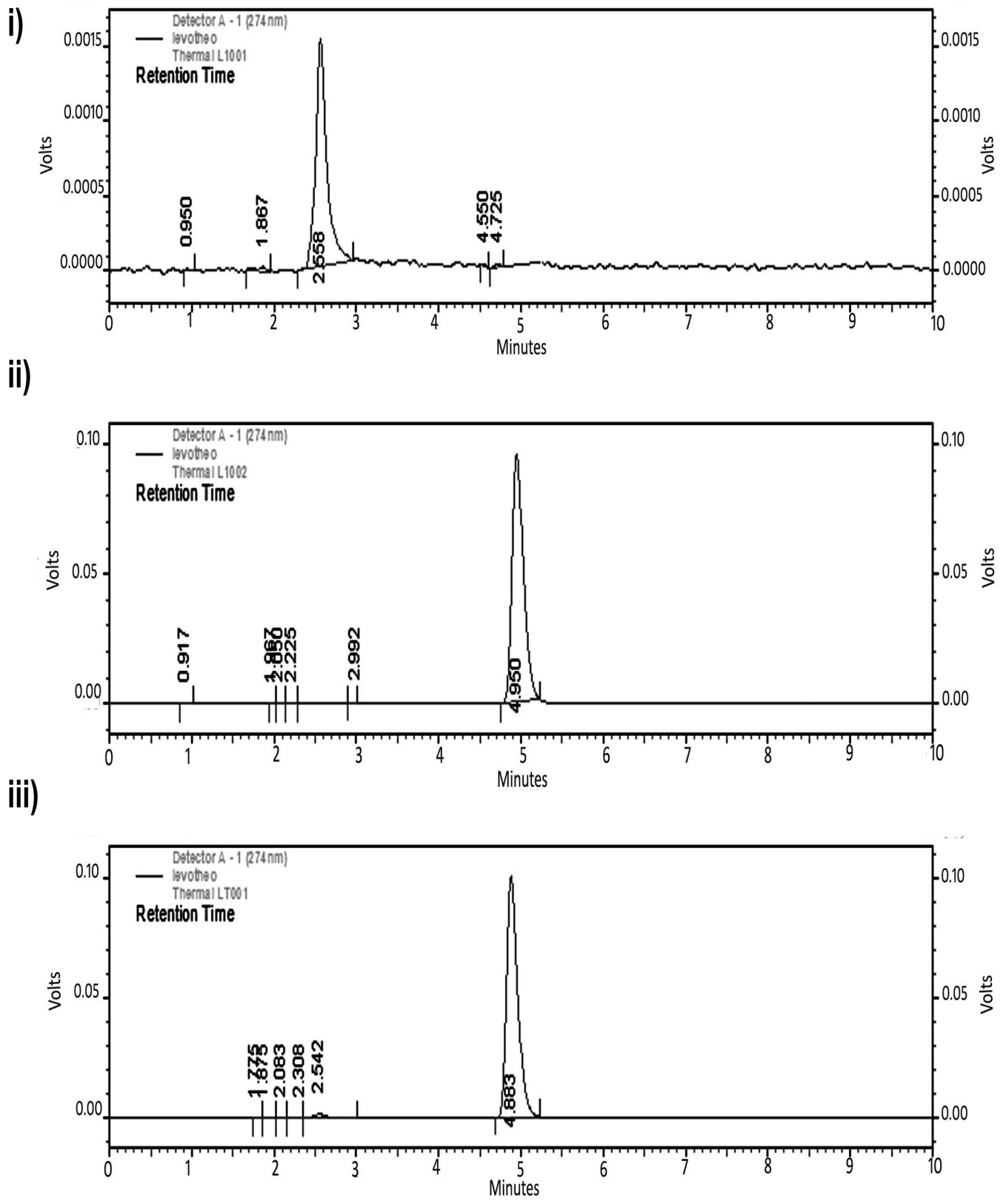

FIGURE 3E - Representative chromatograms of thermally degraded drugs. Each chromatogram represents (i) levosalbutamol sulfate (1 μg.mL^{-1}), (ii) theophylline (10 μg.mL^{-1}), (iii) dosage form having levosalbutamol sulfate (1 μg.mL^{-1}) and theophylline (10 μg.mL^{-1}).

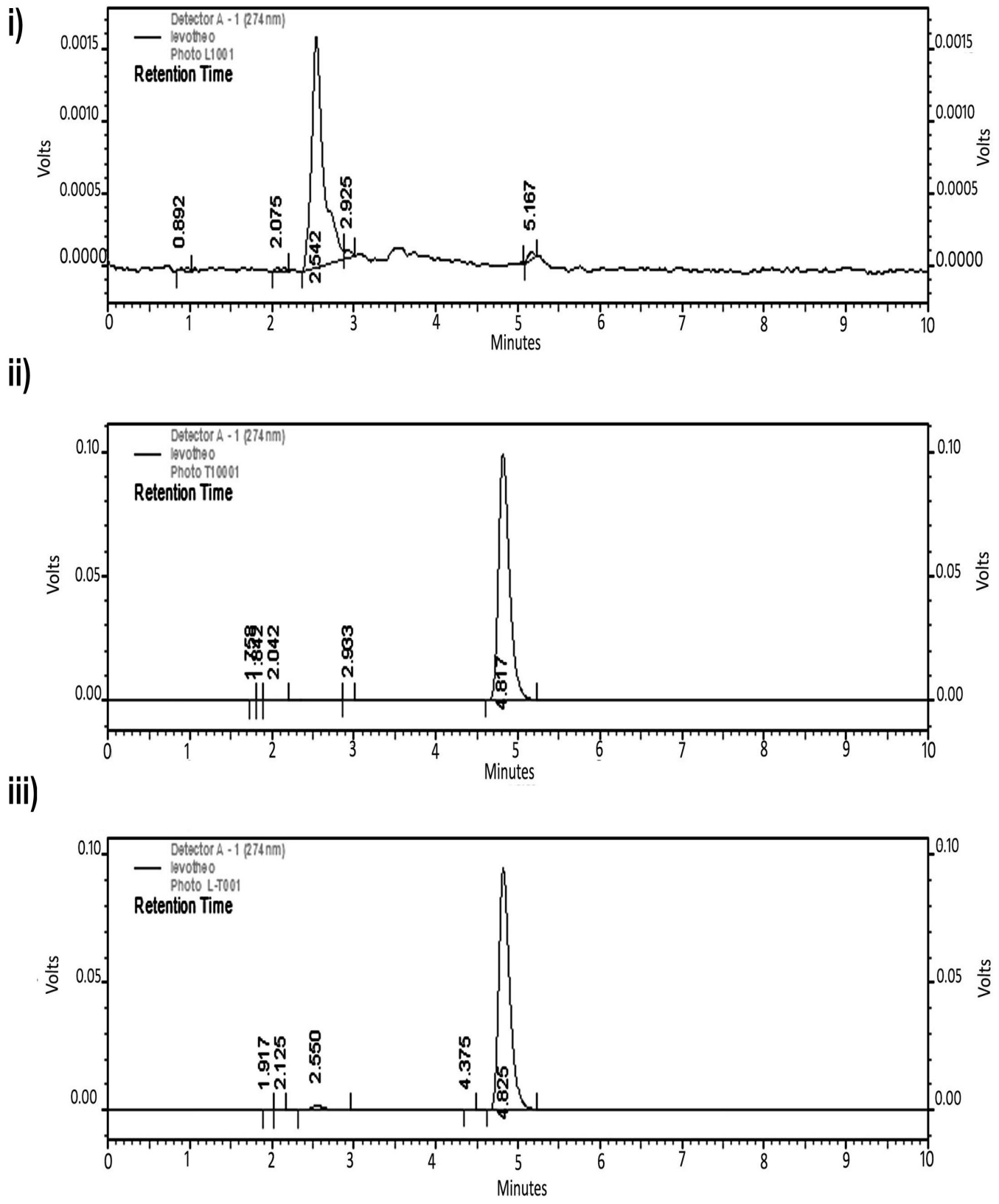

FIGURE 3F - Representative chromatograms of photolysed drugs. Each chromatogram represents (i) levosalbutamol sulfate (1 μg.mL^{-1}), (ii) theophylline (10 μg.mL^{-1}), (iii) dosage form having levosalbutamol sulfate (1 μg.mL^{-1}) and theophylline (10 μg.mL^{-1}).

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