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The determination of a potential impurity in Thalidomide drug substance and product by HPLC with indirect UV detection

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Abstract

Thalidomide molecule, a synthetic derivative of glutamine, can undergo hydrolysis at physiologic pH to form glutamine. Additionally, L-glutamine is one of the starting materials in the synthesis of Thalidomide drug substance. The current USP method for testing glutamine is thin-layer chromatography (TLC) with ninhydrin spray visualization. A more quantitative and automated high performance liquid chromatography (HPLC) method utilizing indirect ultraviolet (UV) detection was developed and validated for the determination of the non-UV absorbing glutamine in Thalidomide drug substance and product. The HPLC mobile phases consisted of phosphoric acid, 2-naphthalenesulfonate sodium and methanol. 2-Naphthalenesulfonate was used as a UV detection probe for glutamine. A segmented isocratic elution program was used to elute glutamine and Thalidomide, respectively. The method was found to be specific for glutamine. The linearity was 0.05–1.25% glutamine with respect to a nominal concentration of 8 mg ml^{-1} Thalidomide sample. The limits of detection and quantitation were found to be 0.03 and 0.05% glutamine, respectively. The injection precision was 2.7% for area responses and 0.2% for the retention times. The recovery of glutamine at three concentration levels was found to be $100.8 \pm 2.8\%$ from placebo and $99.2 \pm 5.8\%$ from spiked Thalidomide drug substances. This newly developed HPLC method was used to determine glutamine in Thalidomide drug substances and products. The results from HPLC were in agreement with those from TLC. Therefore, the method developed is a suitable alternative to the current USP TLC procedure. Additionally, the method offers the advantage of being quantitative and automated.

Keywords: HPLC; Indirect UV detection; Impurity determination; Glutamine; Thalidomide

1. Introduction

Thalidomide (Thalomid) was approved by the FDA in July 1998 for the treatment of erythema nodusum leprosum (ENL) associated with leprosy. Recently, Thalidomide is proving to be a promising drug in the treatment of a number of cancers and inflammatory diseases, such as multiple mye-

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loma [1], inflammatory bowel disease (Crohn's disease) [2–4], HIV and cancer associated cachexia [5,6].

The Thalidomide molecule, a synthetic derivative of glutamine, can undergo hydrolysis at physiologic pH to form glutamine as a degradant [7], Additionally, L-glutamine is one of the starting materials in the synthesis of Thalidomide drug substances [8]. Therefore, glutamine is monitored in both Thalidomide drug substance and pharmaceutical dosage forms. The method currently used to quantitate glutamine in Thalidomide drug substance and products is thin-layer chromatography (TLC) with ninhydrin spray visualization [9]. The TLC method was not automated and only semi-quantitative due to the manual introduction of sample and visual detection. Therefore, it was desired to explore alternative, quantitative chromatographic techniques as potential replacements of TLC for quantitating glutamine in Thalidomide drug substance and products. High performance liquid chromatography (HPLC) is considered to be a more quantitative and automated method than TLC. However, the development of an HPLC method for quantitating glutamine presented two challenging issues. One issue was whether glutamine would have any retention on reversed-phase HPLC. Glutamine is an ammo acid. It is either protonated to form a positive ion at low pH or deprotonated to form an anion at high pH. It also can be present as a zwitterion at pH of its isoelectric point. The second issue is the mode of detection. Since glutamine does not contain chromophores or conjugated double bonds, it cannot be detected directly by the standard UV absorbance techniques.

Glutamine has been determined by using precolumn derivatization for fluorescent or spectrophotometric detection following HPLC and capillary electrophoresis separation [10–16]. Derivatization requires additional sample preparation procedures with the possibility of poor recovery and reproducibility. Ion chromatography with conductivity detection, HPLC with evaporative light scattering detection and LC/MS are also possible alternatives to the determination of glutamine. However, these methods are limited by the availability of these instruments in the laboratory. Indirect UV detection is attractive because such a method can be adapted easily for use with existing HPLC instruments.

Indirect UV detection techniques coupled with the reversed-phase HPLC, ion exchange chromatography and electrophoresis have been used to detect and quantitate some non-UV absorbing analytes [17–27]. Indirect UV detection can be applied to both charged and non-charged analytes. A reagent with UV absorbance (probe) and affinity for the stationary phase is included in the mobile phase. The non-UV absorbing analytes that are injected into the system will give rise to equilibrium disturbance and influence the distribution of the UV absorbing probes. The analytes will give rise to either positive or negative peaks depending on their charges and retention times relative to the probes [28].

In this study, 2-naphthalenesulfonate was used as a UV absorbing probe to detect glutamine at low pH. L-glutamine was used, because this HPLC separation did not recognize the chiral center in the glutamine molecules. This paper presents the development and validation of this method.

2. Experimental

2.1. Materials

Thalidomide drug substances and products were obtained from Celgene Corporation (Warren, NJ), L-glutamine, 2-naphthalenesulfonate sodium (ultra) and N,N-dimethylformamide (DMF, ACS reagent) were purchased from Sigma (St. Louis, MO). Phosphoric acid 85% (ACS reagent) and methanol (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ), All reagents and chemicals were used as received. Water was purified using a Milli-Q-Plus water system (Bedford, Massachusetts).

2.2. Procedures

2.2.1. Preparation of mobile phase

The mobile phase consisted of eluent A and B. Eluent A contained 10 ml methanol mixing with 1000 ml water having 1.5 ml H_3PO_4 and 1 mM 2-

naphthalenesulfonate sodium. Eluent B contained 500 ml methanol mixing with 500 ml water having 1.5 ml H_3PO_4 and 1 mM 2-naphthalenesulfonate sodium. The mobile phase solutions were filtered through a 0.45 μm nylon membrane.

2.2.2. Preparation of glutamine standard solution

Approximately 10 mg of glutamine was accurately weighed and transferred into a 10 ml volumetric flask. About 6 ml of sample solvent (DMF/0.15% H_3PO_4, 1:9 v/v) was added into the flask. The flask was sonicated for 1 min to dissolve glutamine. The solution was then brought to volume with the sample solvent. This was the stock standard solution containing about 1 mg ml^{-1} of glutamine. A 0.01 mg ml^{-1} standard solution was then made by pipetting 1.0 ml of the stock standard solution into a 100 ml volumetric flask and adding the sample solvent to volume. The final nominal concentration of glutamine standard solution was 0.01 mg ml^{-1}.

2.2.3. Preparation of Thalidomide sample solutions

In the testing of Thalidomide drug substances, approximately 80 mg of Thalidomide drug substance was accurately weighed and transferred into a 10 ml volumetric flask, 1.0 ml DMF was pipetted into the flask. The flask was sonicated to dissolve Thalidomide, then a solution of 0.15% H_3PO_4 was added to volume. The sample was sonicated for 1 min. An appropriate amount of Thalidomide product powder equivalent to 80 mg Thalidomide was used in the testing of Thalidomide products.

2.2.4. Chromatographic conditions

The experiments were carried out using a Hewlett–Packard 1100 series HPLC with a variable UV wavelength detector having a 10 mm, 14 μl flow cell. The column was a 150 × 4.6 mm I.D. Agilent Zorbax SB-phenyl with a 5 μm particle size. The injection volume was 100 μl. The detection wavelength was 254 nm. Separation was performed at ambient temperature. The polarity of the signal going to the data acquisition system was reversed so that the analyte peak of interest, glutamine, was positive. A segmented isocratic program was used as below:

Time (min)	A	B	Flow Rate (ml min^{-1})
0	100	0	0.5
10	100	0	0.5
11	0	100	1.5
25	0	100	1.5
26	100	0	0.5
60	100	0	0.5

The first isocratic segment with eluent A was used to elute glutamine. The second isocratic segment with eluent B and a higher flow rate were used to elute Thalidomide.

3. Results and discussions

An HPLC method with indirect UV detection was developed and validated to quantitate the level of glutamine in both Thalidomide drug substances and products. Since glutamine molecules do not contain any chromophores or conjugated double bonds, standard UV detection was not used. Instead, indirect UV detection with 2-naphthalenesulfonate as a UV absorbing probe was used. The mobile phases contained 0.15% H_3PO_4 1 mM 2-naphthalenesulfonate sodium and methanol. The chromatographic conditions were optimized for the amounts of 2-naphthalenesulfonate sodium, H_3PO_4 and methanol in the mobile phases.

When the concentration of 2-naphthalenesulfonate was lower than 1 mM, glutamine peak eluted closer to the front solvent peak 2. When the concentration of 2-naphthalenesulfonate was increased to 1.5 mM, the detection sensitivity decreased significantly as observed [29]. The decrease in the sensitivity resulted from the substantial increase in the baseline noises due to a higher concentration of the UV absorbing probe in the mobile phase. The baseline was so noisy that glutamine could not be detected at a concentration of ten times of the standard solution.

About 0.15% H_3PO_4 was used to maintain a low mobile phase pH and to reduce the effects of free silanols on the stationary phase. The pH of the solution of 0.15% H_3PO_4 and 1 mM 2-naphthalenesulfonate sodium was measured to be 2.09 ± 0.05. Therefore, glutamine ($pK_{a1} = 2.17$ $pK_{a2} = 9.13$) [30] was present as a cation in eluent A, and 2-naphthalenesulfonate was present as an

anion. Glutamine would have a larger retention time in a 100% aqueous solution. However, a small amount of methanol in eluent A was used to prevent the stationary phase from collapsing in a 100% aqueous mobile phase. The column efficiency was not changed after more than 20 l of eluents A and B were pumped through the column.

The sample solvent contained DMF/0.15% H_3PO_4 (1:9, v/v). A higher amount of DMF in the sample solvent caused the peak shape of glutamine to distort, because the eluent A was very weak and the injection volume was large in order to increase the detection sensitivity for glutamine. Additionally, DMF itself gave rise to a peak that was close to glutamine. It was observed that DMF at a concentration above 10% in the solvent generated a large peak that interfered with the glutamine peak. However, sample solvent required 10% DMF in order to dissolve Thalidomide, because it has a very low solubility in the aqueous solution. The solubility of Thalidomide in water is about 50 μg ml^{-1} [30]. Therefore, Thalidomide drug substance was first dissolved completely in 1.0 ml DMF in a 10 ml volumetric flask under sonication; then a solution of 0.15% H_3PO_4 was added to volume. This solution was further sonicated for 1 min to extract and dissolve only glutamine. The mixture was then filtered and the solution was injected onto the HPLC. The sample preparation procedures were validated by spiking Thalidomide drug substance with glutamine standard solutions. The recoveries of glulamine from 100% Thalidomide drug substance are presented in Section 3.5.

The performance of the HPLC method with indirect UV detection was validated with respect to specificity, limits of detection and quantitation, system precision, linearity, recovery and robustness. Additionally, this newly developed HPLC method was used to determine glutamine in Thalidomide drug substance and products. The results of these studies are discussed below.

3.1. Specificity

A Thalidomide solution was spiked with a glutamine standard solution. Glutamine was separated from Thalidomide and the other components in the solvent as shown in Fig. 1. Peaks 1, 2, 4 and 5 in Fig. 1a were from the sample solvent. Peak 3 was glutamine. In Fig. 1b, peaks 6 and 7 were system peaks that appeared with the same retention times and response patterns in all the chromatograms in this HPLC system. Peak 8 was Thalidomide with a negative response. Thalidomide has UV absorbance and gave rise to a positive peak. However, the polarity of the signal going to the data acquisition system was reversed, so the Thalidomide peak became negative.

The chromatograms consisted of two isocratic segments in Fig. 1. The first segment ranging from 0 to 10 min was where glutamine eluted and the

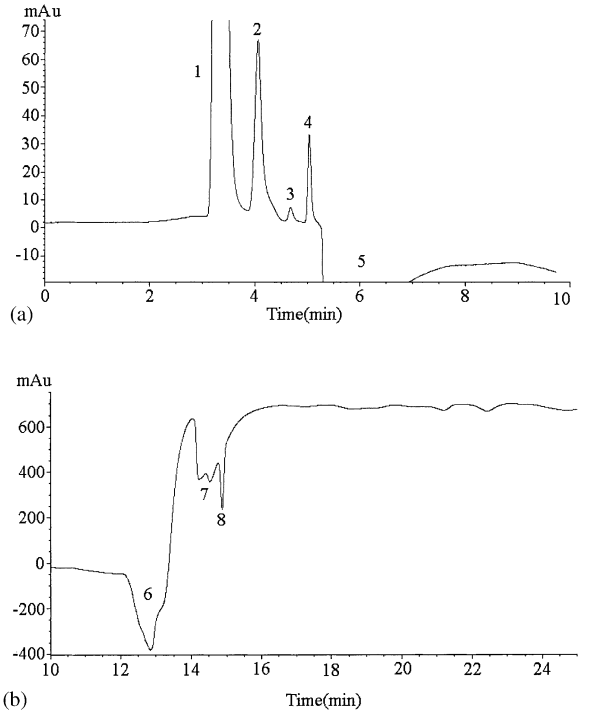

Fig. 1. (a) Chromatogram of Thalidomide drug substance spiked with a 0.01 mg ml^{-1} glutamine standard solution from 0 to 10 min. Peaks 1, 2, 4 and 5 are from the solvent. Peak 3 is glutamine. Eluent A, 1000 ml, 1×10^{-3} M 2-naphthalenesulfonate sodium in 0.15% phosphoric acid (85%), 10 ml methanol. Eluent B, 500 ml, 1×10^{-3} M 2-naphthalenesulfonate sodium in 0.15% phosphoric acid (85%), 500 ml methanol. Column, Zorbax SB-phenyl 150 × 4.6 mm, 5 μm particle size. (b) Chromatogram of Thalidomide drug substance spiked with 0.01 mg ml^{-1} glutamine standard solution from 10 to 25 min. Peaks 6 and 7 are the system peaks. Peak 8 is Thalidomide. System as in Fig. 1a.

second segment ranging from 10 to 25 min was where Thalidomide eluted. The two isocratic segments were used because Thalidomide and glutamine have a large difference in the retention times. Additionally, the baseline in the second isocratic segment was 600 mAu higher than it was in the first isocratic segment. The reason was that eluent B contained less of the UV absorbing probe 2-naphthalenesulfonate than eluent A. The baseline accurately drifted down. However, the polarity of the signal was reversed, so the baseline was shown to drift up in the second segment. Therefore, it was not possible to show both glutamine and Thalidomide in the same scale. After 25 min the system was equilibrated with the initial eluent A and this part of the chromatogram was not shown. In this HPLC system, the mobile phase contained a UV absorbing reagent that require sometimes to break through completely (the concentration of the UV probe in the effluent equals to that in the influent). It was just like a frontal chromatography of the UV probe after 25 min with the eluent A. Therefore, a longer equilibrium time was needed for the baseline to return to 0. Fig. 2a and b shows the chromatograms of the solvent for these two isocratic segments, respectively.

Besides glutamine, Thalidomide also has ten other related compounds or potential degradants. Those compounds can be divided into two groups according to their UV absorbance characteristics. One group has eight potential degradants with UV absorbance. They are *N*-phthaloyl and *N*-(o-carboxybenzoyl) derivatives as well as phthalic acid and α-aminoglutarimide. They all eluted at 12–16 min with negative peaks as Thalidomide did. Fig. 3 shows the chromatograms of the penultimate synthetic intermediate *N*-phthaloyl-DL-glutamine (NPG) and the potential primary hydrolysis product *N*-(o-carboxybenzoyl)-DL-glutarimide (CGI). All of the related compounds in this group did not interfere with glutamine. These compounds were tested by the USP method [9].

The other group, including iso-glutamine and glutamic acid, does not have chromophores or conjugated double bonds. Like glutamine, they cannot be detected with the standard UV detection. They also are very similar to glulamine in

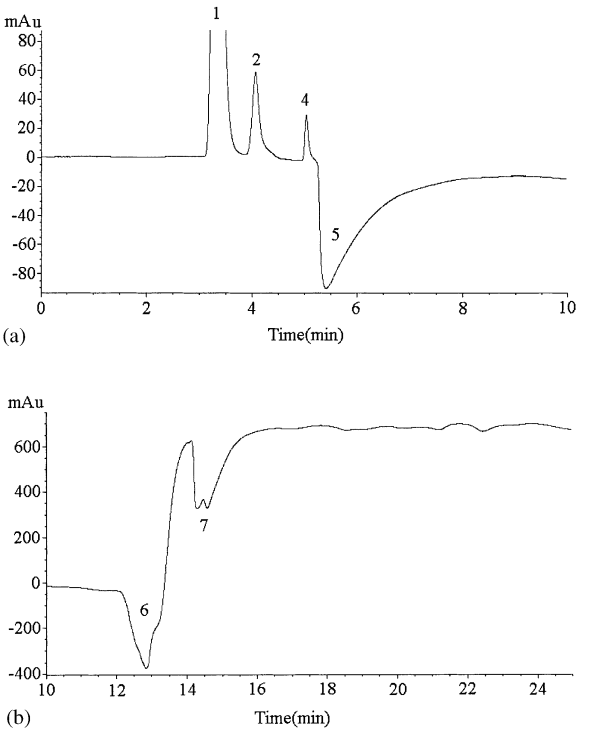

Fig. 2. (a) Chromatogram of the solvent from 0 to 10 min. Peaks l, 2, 4 and 5 are from the solvent. System as in Fig. 1a. (b) Chromatogram of the solvent from 10 to 25 min. Peaks 6 and 7 are the system peaks. System as in Fig. 1a.

their structures. Therefore, the specificity of this method was investigated in the presence of iso-glutamine and glutamic acid. Fig. 4 presents the chromatograms of glutamine, iso-glutamine and glutamic acid in a solution of 0.15% H_3PO_4 at 0.1 mg ml^{-1} that was ten times the standard solution

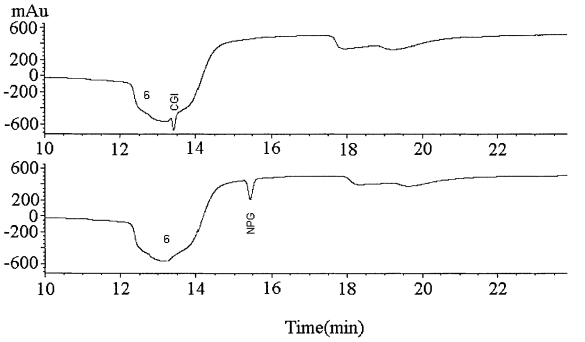

Fig. 3. Chromatograms of 0.2 mg ml^{-1} standard solutions of NPG and CGI. Peak 6 is system peak. System as in Fig. 1a.

concentration of glutamine. Fig. 5 shows the chromatogram of a solution of glutamine and glutamic acid at 0.01 mg ml^{-1} or 0.1% of the working concentration. The resolution between glutamine and glutamic acid was 1.5 in Fig. 5. Therefore, glutamine acid was separated from glutamine. Iso-glutamine was not included in Fig. 5 because only glutamic acid eluted closely to glutamine as shown in Fig. 4. Glutamic acid and iso-glutamine would co-elute with the solvent peaks 4 and 5 that were associated with DMF. Therefore, DMF was not used in these solutions to demonstrate that glutamic acid and iso-glutamine were separated from glutamine. The studies indicated that this HPLC method with indirect UV detection is specific for the determination of glutamine in Thalidomide drug substances and products.

3.2. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD was determined to be 2.54 μg ml^{-1}, or 0.03% glutamine. At this concentration, the signal to noise ratio was 4 based on the noise level at the 7.5–8.5 min range. The LOQ was determined to be 4.0 μg ml^{-1}, or 0.05% glutamine. The relative standard deviation (R.S.D.) of the areas from the consecutive six injections was 8.3%. An R.S.D.% of 10 is usually accepted at the LOQ level. The areas of the six injections are shown in the second column in Table 1.

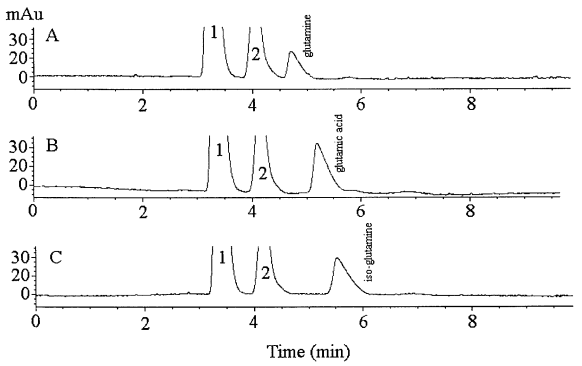

Fig. 4. A, 0.1 mg ml^{-1} glutamine; B, 0.1 mg ml^{-1} glutamic acid; C, 0.1 mg ml^{-1} iso-glutaminc. System as in Fig. 1a.

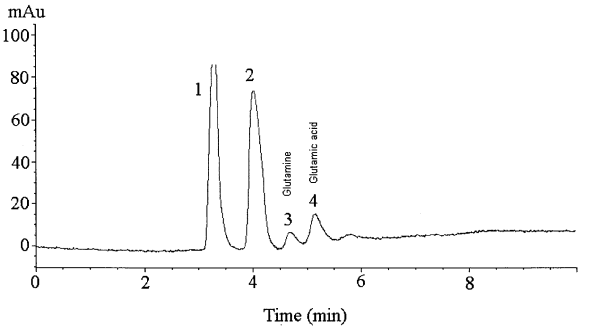

Fig. 5. Chromatogram of 0.01 mg ml^{-1} glutamine and glutamic acid. Peaks 1 and 2 are from the solvent. Peak 3 is glutamine. Peak 4 is glutamic acid. System as in Fig. 1a.

3.3. System precision

The system precision was determined by injecting a standard solution of glutamine at 0.0101 mg ml^{-1} six times. This concentration represented a 0.1% glutamine level. The R.S.D. for the peak areas was 2.7%. The R.S.D. for the retention times was 0.2%. The areas and retention times for each injection are also shown in the third and fourth columns, respectively, in Table 1.

3.4. Linearity

The linearity of the method was evaluated over the glutamine concentrations ranging from 0.004 to 0.10 mg ml^{-1}. These concentrations corresponded to 0.05% (LOQ)–1.25% glutamine. Six concentration levels were prepared and injected twice for each level onto HPLC. Fig. 6 shows the linearity results. The linear regression analysis of the peak areas versus the concentrations resulted in a correlation coefficient (R^2) of 0.9999 with a slope of 2535.2 ± 20.6 and an intercept of -1.088 ± 1.011 at the 95% confidence interval. The intercept corresponded to a −4% response at 0.1% glutamine. The specification limit of glutamine is 0.1% [9]. Therefore, the linearity range is suitable for the quantitation of glutamine in the Thalidomide drug substances and products.

3.5. Recovery from Thalidomide drug substance

The recovery was determined by spiking Thalidomide drug substance with glutamine standard

Table 1
LOQ and system precision

Analysis	LOQ area (mAU*S)	System precision area (mAU*S)	System precision retention time (min)
1	8.25234	18.54323	4.494
2	8.58453	18.60275	4.479
3	8.14473	17.30381	4.480
4	9.38122	18.42579	4.482
5	7.40443	18.31316	4.478
6	7.75185	18.63391	4.465
Average	8.25318	18.30378	4.480
R.S.D. (%)	±8.3	±2.7	±0.2

solutions at 0.0625, 0.100 and 1.25% of the working concentration. An accurate weight of 80 mg Thalidomide drug substance was dissolved in 1.0 ml DMF in a 10 volumetric flask. This solution was spiked with a 1.0 ml glutamine solution. A solution of 0.15% H_3PO_4 was added to volume. Three samples were prepared at each level and injected twice each. The average recovery from the nine samples was 99.2±5.8%. The results of each individual recovery, the mean and the R.S.D.% are shown in Table 2.

3.6. Recovery from the placebo of Thalidomide drug product

The recovery of glutamine from the placebo was determined by spiking the placebo with glutamine standard solutions at 0.0625, 0.100 and 1.25% of the working concentration. An accurate weight of 350 mg placebo was transferred into a 10 ml volumetric flask. A solution of glutamine was added to volume. Three samples were prepared at each level and injected twice each. The average recovery from the nine samples was 100.8±2.8%. The results of each individual recovery, the mean and the R.S.D.% are shown in Table 2.

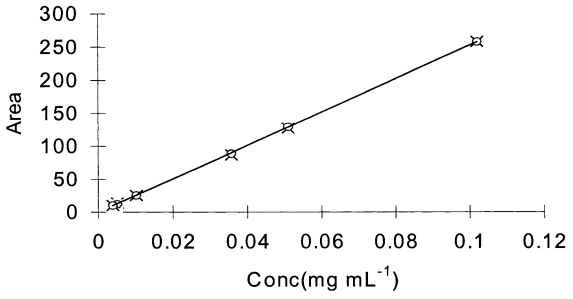

Fig. 6. Linearity of glutamine, O predicted points, *x* experimental points.

3.7. Method robustness

The robustness was studied by deliberately changing the flow rate by ±20% in the first isocratic segment where glutamine eluted. Additionally, the amount of methanol in eluent A was changed by ±20%, because glutamine eluted in eluent A. The concentrations of H_3PO_4 and 2-naphthalenesulfonate sodium were changed by ± 33 and ±50%, respectively. A standard solution was used in this study. The chromatographic merits were evaluated in terms of the retention times and resolutions. The results are presented in Table 3. The resolution between the front solvent peak 2 and glutamine was resolution 1; and the resolution between glutamine and the back solvent peak 4 was resolution 2.

When the flow rate decreased or increased by 20%, the retention times increased or decreased by 25 and 15%, respectively. The retention times did not change significantly in the other conditions. When the concentration of 2-naphthalenesulfonate was increased to 1.5 mM, glutamine was not detected due to the baseline noises resulted from the UV absorbing probe. When the concentration of 2-naphthalenesulfonate was decreased to 0.5 mM, glutamine eluted closer to the front solvent peak 2 with the resolution 1 of 1.30. When the concentration of H_3PO_4 was increased to 0.2%, the back solvent peak 4 eluted closer to glutamine.

Table 2
Recovery from placebo and Thalidomide drug substance

Recovery from Thalidomide			Recovery from placebo		
Added (μg ml^{-1})	Found (μg ml^{-1})	Recovery (%)	Added (μg ml^{-1})	Found (μg ml^{-1})	Recovery (%)
5.415	4.963	91.7	5.415	5.627	103.9
5.415	5.910	109.1	5.415	5.545	102.4
5.415	5.756	106.3	5.415	5.665	104.6
10.94	11.03	100.8	10.14	10.02	98.8
10.94	10.68	97.6	10.14	10.33	101.9
10.94	11.01	100.6	10.14	10.19	100.5
108.3	107.2	99.0	114.8	116.5	101.5
108.3	103.1	95.2	114.8	111.8	97.4
108.3	100.6	92.9	114.8	110.7	96.4
x±S.D.		99.2±5.8			100.8±2.8
R.S.D. (%)		5.8			2.8

The resolution 2 was smaller than 1.0. The resolutions were all acceptable in the other conditions. Generally, the method conditions gave the best separation.

3.8. Analysis of glutamine in Thalidomide drug substances and products

A batch of Thalidomide drug substance and product was analyzed using this newly developed method. A close-up chromatogram is shown in Fig. 7. Glutamine was not detected in both Thalidomide drug substance and product as shown in Fig. 7a. The results indicated that gluamine was not present in these batches of Thalidomide drug substances and products. These results are in agreement with those from TLC analysis. Fig. 7b shows the corresponding second segment where Thalidomide eluted. Although glutamine was not detected in the Thalidomide drug substances and products, the validation studies have demonstrated that this newly developed HPLC method with indirect UV detection is specific, sensitive, accurate and precise to quantitate glutamine in Thalidomide drug substances and products.

4. Conclusions

An HPLC method with indirect UV detection was developed and validated for the determination of the impurity glutamine in Thalidomide drug

Table 3
Method robustness

Parameters	Values	Retention time (min)	Resolution 1	Resolution 2
Flow rate (ml min^{-1})	0.4	5.640	2.29	1.87
	0.5[a]	4.477	2.24	1.51
	0.6	3.790	2.16	1.74
Methanol (ml) in 1000 ml H_2O	12.0	4.483	1.94	1.59
	10.0[a]	4.477	2.24	1.51
	8.0	4.520	2.27	2.34
85% H_3PO_4 (ml) in 1000 ml H_2O	2.0	4.422	2.27	0.796
	1.5[a]	4.477	2.24	1.51
	1.0	4.467	1.54	1.50
2-naphthalenesulfonate sodium (mM) in 1000 ml H_2O	0.5	4.334	1.30	2.12
	1.0[a]	4.477	2.24	1.51
	1.5	NA	NA	NA

[a] Method conditions.

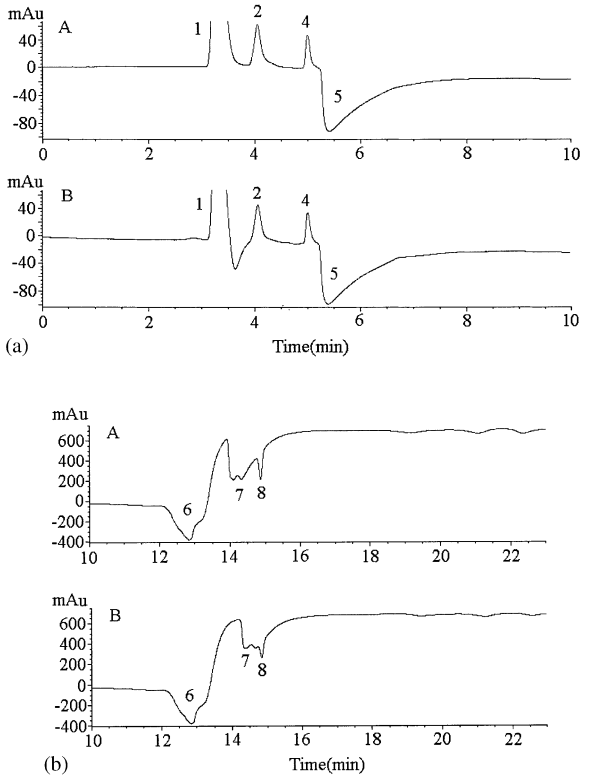

Fig. 7. (a) A, Chromatogram of Thalidomide drug substance from 0 to 10 min; B, Chromatogram of Thalidomide drug product from 0 to 10 min. Peaks 1, 2, 4 and 5 are from the solvent and placebo, respectively. System as in Fig. 1a. (b) A, Chromatogram of Thalidomide drug substance from 10 to 23 min, B: Chromatogram of Thalidomide drug product from 10 to 23 min. Peaks 6 and 7 are the system peaks. Peak 8 is Thalidomide. System as in Fig. 1a.

substances and products. The method was found to be specific, precise, accurate and sensitive. Therefore, this method is a suitable alternative to the current USP TLC procedure. Additionally, the method offers the advantage of being quantitative and automated.

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