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Short communication

Validation of a HPLC method for the quantification and purity determination of SK3530 in drug substance and tablet

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Abstract

SK3530·2HCl, (2-(5-(4-(2-hydroxyethyl)piperazin-1-ylsulfonyl)-2-*n*-propoxyphenyl)-5-ethyl-7-*n*-propyl-3,5-dihydro-4H-pyrrolo[3,2-d]pyrimidin-4-one dihydrochloride), is a novel a new phosphodiesterase type V (PDE V) inhibiting agents. The pharmaceutical development of SK3530 necessitated the availability of an assay for the quantification and purity determination of SK3530 active pharmaceutical ingredient (API) and its pharmaceutical dosage form. A reversed-phase high performance liquid chromatographic (HPLC) method with ultraviolet (UV) detection was developed, consisting of separation on a C18 column with a CapcellPack MG (4.6 mm × 150 mm, 5 μm) column with ammonium acetate buffer (pH 4.0, 20 mM)–acetonitrile (60:40, v/v) as the isocratic mobile phase and UV detection at 250 nm. The method has been shown good chromatographic separation for SK3530 and the other related substances. The method was found to be linear 200–300 μg/ml, precise and accurate. Stress testing showed degradation products, which were well separated from the parent compound, confirming its stability-indication capacity. Moreover, the use of LC–MS and on-line diode array detection enabled us to propose structures for degradation products.

Keywords: SK3530; HPLC; Degradation products; Mass spectrometry; Diode array detection

1. Introduction

SK3530·2HCl, 2-(5-(4-(2-hydroxyethyl)piperazin-1-ylsulfonyl)-2-*n*-propoxyphenyl)-5-ethyl-7-*n*-propyl-3,5-dihydro-4H-pyrrolo[3,2-d]pyrimidin-4-one dihydrochloride, is a new phosphodiesterase type V (PDE V) inhibitor which was developed for the treatment of male erectile dysfunction (Fig. 1). The pK_a of SK3530 was 5.99. The log partition coefficient (octanol/water) of SK3530 was 3.67. The tablets containing SK3530·2HCl are currently in clinical trials for the treatment of male erectile dysfunction.

The pharmaceutical development of an oral dosage form for SK3530 necessitated the availability of an assay [1–6] for quantification and purity determination of SK3530 and its tablet. Earlier chromatographic methods for SK3530 utilized two separate isocratic, reversed-phase systems: one for a quantitation of impurities and another for an assay of SK3530. These methods included phosphate buffer as the mobile phase. Therefore, it is incompatible with mass spectrometric detection. Compatibility with mass spectrometric detection can be useful when identification of unknown impurities is required.

This paper describes the development of a reversed-phase HPLC method with ultraviolet detection (UV) that is suitable as a stability-indicating for the determination of SK3530 and its impurities. Validation of the analytical method was performed according to the international guideline [3,4]. On-line diode array detection (DAD) and HPLC coupled to mass spectrometry (LC/MS) were used to attain more information on the observed impurities and degradation products of SK3530.

2. Experimental

2.1. Chemicals

SK3530·2HCl (API), SK3530 were obtained from research laboratory of SK Chemicals Co. Ltd. (Suwon, Korea). HPLC-grade acetonitrile was purchased from Burdic & Jakson (MI,

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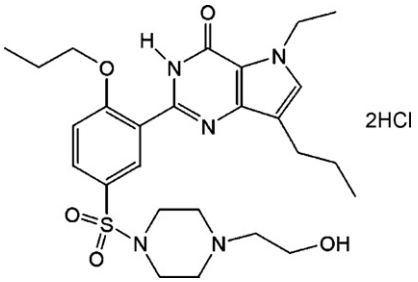

Fig. 1. Chemical structures of SK3530.

USA). Reagent grade ammonium acetate was purchased from Sigma Chemical Co. Ltd. (Mo, USA). Reagent grade glacial acetic acid was purchased from Shinyo Pure Chemicals (Osaka, Japan). The inactive ingredients used as the drug-matrix include, microcrystalline cellulose type PH 102 of NF grade (Mingtai, Taiwan), croscarmellose sodium of NF grade (FMC, Ireland), colloidal silicon dioxide of NF grade (Tomita, Japan) and magnesium stearate of NF grade (Merck, USA), Opadry orange (Colorcon, UK) used as a film material was composed of polyvinyl alcohol, talc, titanium dioxide, FD&C yellow No. 6 lake, lecithin, FD&C blue No. 2 lake and iron oxide red.

The compounds related to SK3530 which could be expected as impurities or might appear as degradation products have been prepared and identified at SK chemicals Co. Ltd. (Suwon, Korea). These compounds include, 3-(5-ethyl-4, 5-dihydro-4-oxo-7-*n*-propyl-3H-pyrrolo[3,2-d]pyrimidin-2-yl) -4-propoxybenzenesulfonic acid (Impurity A); 5-ethyl-2-[5-piperazinylsulfonyl-2-*n*-propoxyphenyl]-7-*n*-propyl-3,5-dihydro-4H-pyrrolo[3,2-d]pyrimidin-4-one (Impurity B); 5-ethyl-2 {5-[4-(2-hydroxyethyl)piperazin-1-ylsulfonyl]-2-hydroxyphenyl}-7-*n*-propyl-3,5-dihydro-4H-pyrrolo[3,2-d]pyrimidin-4-one (Impurity C); 5-ethyl-2-[2-*n*-propoxy-5-(4-*n*-propylpiperazin-1-ylsulfonyl)phenyl]-7-*n*-propyl-3,5-dihydro-4H-pyrrolo [3,2-d]pyrimidin-4-one (Impurity D). The structural formulas of Impurities A, B, C and D are shown in Fig. 2.

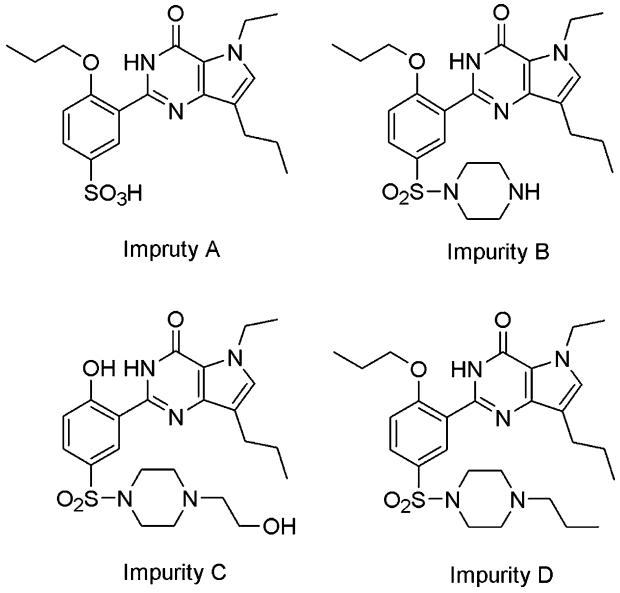

Fig. 2. Chemical structures of Impurities A, B, C and D.

High pure water was prepared by using Milli-Q plus purification system from Millipore Co. (MA, USA).

3. Methods

3.1. Sample preparation

3.1.1. Standard solutions of SK3530 and related substances

Samples of about 100 mg SK3530 or related substances (Impurities A, B, C and D) were accurately weighed quantity of was dissolved in the mobile phase and diluted quantitatively. Serial dilutions were carried out, using the mobile phase, to obtain solutions of known concentrations to be used for the standard preparation and the assay purposes. The solutions were filtered through a PTFE 0.45 μm membrane filter (Sun Sri Co., NC, USA). This standard solution was stored at 4 °C until use.

3.1.2. Standard solution of SK3530 and related substances in the drug-matrix

Samples of about 100 mg of SK3530 or related substances were accurately weighed and mixed with appropriate proportions (1:3, w/w) of the drug-matrix components mentioned above. The mixtures were dissolved and diluted quantitatively to 100 ml using the mobile phase. The solutions were sonicated for 15 min and were filtered through a PTFE 0.45 μm membrane filter. The filtrate was used to prepare solutions of various quantities of SK3530 using the mobile phase as a diluent. Samples were stored at 4 °C until use.

3.1.3. Preparation of sample solution from API and tablet containing it

An API sample was prepared by accurately weighing 56.9 mg of API and dissolving in 200 ml of the mobile phase, corresponding to 250 μg/ml SK3530. Samples were stored at 4 °C until use.

An SK3530 tablet was prepared with API and excipients, as above mentioned. SK3530 tablet and placebo tablet were prepared. To prepare sample solution from the tablets, 20 tablets were milled to a fine powder. The powder, equivalent to about 50 mg of SK3530, was dissolved in 200 ml of mobile phase. The solution was filtered through a PTFE 0.45 μm membrane filter.

3.2. Chromatographic conditions

3.2.1. HPLC

The HPLC system consisted of HP1100 series (Agilent Technologies, Palo Alto, USA): G1379 on-line degasser, G1312A binary pump, G1329A auto-sampler, G1330B thermostated autosampler, G1316A column oven and G1315B diode-array detector. Data was acquired and processed with Chemstation® (Agilent Tenchologies). The mobile phase was the mixture of 0.02 M ammonium acetate buffer (pH 4.0) and acetonitrile (60:40, v/v). Its flow rate was 1.0 ml/min. Separation was achieved using a CapcellPack MG column (4.6 mm × 150 mm, 5 μm particle size, C18, Shiseido, Japan). The column temperature was kept at 40 °C. The detection wavelength was 250 nm.

The injection volume was 10 μl. Concentration of the API was calculated using the peak area of sample solutions compared to that of standard solution.

3.2.2. LC/MS

HPLC conditions were described in Section 3.2.1. The eluate was led into the interface of an LCQ-DECA XP plus ion-trap mass spectrometer. The instrument was equipped with an electro-spray ionization (ESI) source (Thermo Finnigan, USA), controlled by the Xcaliber®.

The mass spectrometric conditions were as follows: ESI positive ionization mode, auxiliary gas was 10 arb, spray voltage was 5.0 kV, full scan from 1500 to 200 amu. In MS/MS determination, collision energy (CID) was set at 27%, the mass detector range was set from 1500 to 200 amu limited by the instrument.

For direct injection MS analysis, capillary temperature was 275 °C. Sheath gas was 60 arb. For LC/MS analysis, capillary temperature was 275 °C. Sheath gas was 60 arb. The effluent was split in a ratio of 2:8 before being pumped into the mass spectrometer.

3.3. Validation procedure

The HPLC method was validated with respect to the following parameters: specificity, linearity, accuracy, precision, and stability of sample solution [3–6].

3.3.1. Specificity

Two types of specificity experiments were performed. In the first experiment, interference between API and impurities, including tablet excipients, were evaluated from the comparison of spectral purity and mass spectra obtained from the analysis for the API solution and sample solutions containing API, other impurities and tablet excipients. In the second experiment, chromatographic purities were also evaluated in the presence of possible degradation products which were generated by several accelerated conditions. Stock solutions of 2 mg/ml were subjected to several accelerated conditions [7]. Heat: 1 ml of API stock solution and API itself was exposed to 100 °C for 24 h. Oxidation: to 1 ml of API stock solution, 1 ml of 0.3% hydrogen peroxide solution was added and storing the sample at 100 °C for 90 min. Acidic or alkaline condition: to 1 ml API stock solution 0.5 ml of 3 M hydrochloric acid (HCl) or 3 M sodium hydroxide (NaOH) was added and storing the sample at 100 °C for 24 h. Samples were neutralized using 0.5 ml of 3 M NaOH or 3 M HCl. All samples were diluted with mobile phase to a theoretical concentration of 250 μg/ml SK3530 before analysis.

3.3.2. Linearity

In order to evaluate the linearity of HPLC assay method, calibration curves (five concentration of standard solution versus peak area) were constructed for SK3530 and its related substances. Calibration curves were also plotted in the presence of tablet component.

The degree of linearity was assessed by the correlation coefficient, *y*-intercept, slope, the confidence intervals for the slope and the intercept of the regression line [8–11].

3.3.3. Accuracy and precision

In order to estimate the accuracy of the assay method, different sample solutions of SK3530 and SK3530 with other impurities at various concentration levels were analyzed in the pure solutions state. The same sample solutions in the existence of tablet excipients were also analyzed [8,9].

In relation to the precision, system precision and intermediate precision were investigated. To assess the system precision,

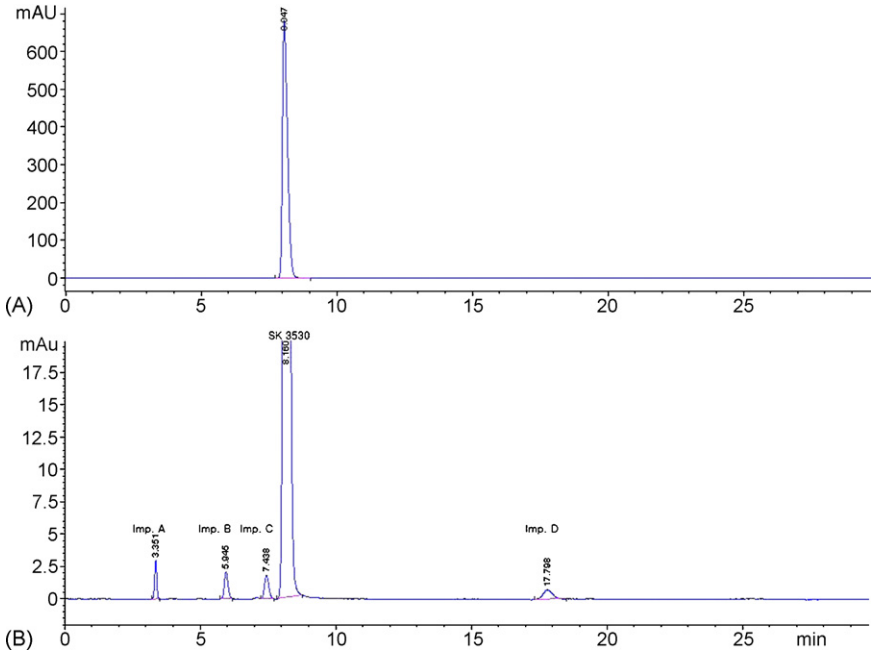

Fig. 3. HPLC chromatograms for SK3530 in standard preparation (Chromatogram A), a mixture of SK3530 and related compounds prepared in the drug-matrix solution (Chromatogram B).

0.05 and 100% sample solutions for SK3530 were injected 10 times consecutively and the R.S.D. (%) for 10 peak areas was calculated. Intermediate precision was evaluated by assaying quality control samples in triplicate in three separate runs, with within-run and between-run. The within-run and between-run precisions were calculated by analysis of variance (ANOVA) for each test concentration using the analytical run as the grouping variable [7]. From the ANOVA analyses, the day mean square (DayMS), error mean square (ErrMS) and grand mean (GM) were obtained. Within-run and between-run precisions were defined using Eqs. (1) and (2), respectively, where n is the number of replicates

$$\text{within-run\_precision}\,(\%) = 100 \times \frac{\sqrt{\text{ErrMS}}}{\text{GM}} \quad (1)$$

$$\text{between-run\_precision}\,(\%) = 100 \times \frac{\sqrt{(\text{DayMS} - \text{ErrMS})/n}}{\text{GM}} \quad (2)$$

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

Limit of detection was calculated as three times of noise level from the calibration curve of SK3530 and its related compounds. Limit of quantitation was calculated as ten times of noise level from the same chromatogram [4].

4. Results and discussion

4.1. Specificity

The HPLC chromatogram recorded for the matrix (mixture of the excipients) solution showed no peaks within 30 min of run time. Fig. 3 shows a representative chromatogram for SK3530 alone (Chromatogram A) and a chromatogram (Chromatogram B) for a mixture of SK3530 with its related compounds A, B, C and D (a mixture containing about 2.5 μg/ml of each component) in the drug-matrix solution. The figure shows that SK3530 is clearly separated from its related compounds and these four compounds are also well separated from each other. Thus, the HPLC method presented in this study is selective for SK3530 and the other four related compounds, which might coexist as impurities originated from the synthesis process.

The specificity was also demonstrated by the accelerated degradation of API as was described in Section 3.3.1. Table 1 shows the relative peak areas of SK3530 and additional impurities or degradation products observed during accelerated testing. Degradation products were included in the table only when their relative peak areas (degradation peak area per total peak area) were higher than 0.15% in one of the analyzed samples.

Only minor degradation was observed after subjecting SK3530 stock solution to acidic, alkaline and heat stress conditions at solution state for 24 h. The parent peak was well separated form the all of peaks from the accelerated degradation. However, the decomposition was significant when SK3530·2HCl was treated with 0.3% H_2O_2 or heated at solid state where the SK3530 recovery was 92.97 and 98.17% and the total degradation was 7.03 and 1.83%, respectively. The parent peak was also well separated from degradation products generated by heat and oxidation.

Samples were analyzed against a control sample (no degradation treatment). Degradation peaks, where observed, were resolved from the SK3530 peak. The results showed that, when each of the assayed substances was decomposed with the accelerated degradation test, the decomposition products did not interfere with the determination of the primary substances (Fig. 4). UV Spectral extraction for the peak of SK3530 was carried on at the time of upslope, apex and down-slope and comparison among three UV spectra shows that there is no co-elute within retention time of SK3530. LC/MS analysis also showed no additional ions, which is conformational evidence that there is no co-eluting material with SK3530 in this HPLC method.

Because degradation products are susceptible to positive ionization, these products are well detected in the positive mode. The major degradants (compounds 3, 8 and SK3530) was appeared the most abundant m/z values and their corresponding MS/MS fragments observed in the ion-trap. The formation of dimmers was also observed from corresponding m/z value.

An overlay chromatography of the degradation study was shown in Fig. 4. The UV spectra of the difference degradation

Table 1
Relative peak areas (%) of SK3530, impurities and degradation products in SK3530 API and after API in solution to oxidation, heat (solution and solid), acidic and alkaline condition compared to SK3530 reference standard

Compound	RRT	Ref	API	Acidic	Alkaline	Oxidation	Heat (solution)	Heat (solid)
1	0.15					4.44		
2	0.36					0.59		
3	0.39			0.69				0.45
4	0.43					0.36		
5	0.46					0.52		
6	0.57					0.33		
7	0.66				0.15	0.31		
8	0.88			0.16	0.53	0.23		1.38
9 (SK3530)	1.00	100.0	100.0	99.15	99.32	92.97	100.0	98.17
10	1.09					0.25		

RRT: relative retention time.

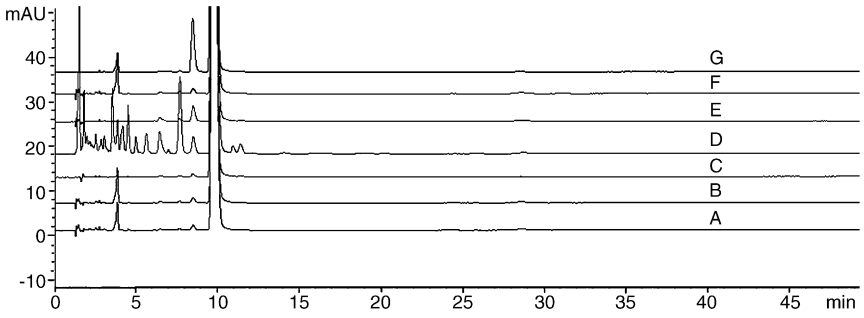

Fig. 4. Overlay of chromatograms of SK3530 degradation study chromatograms. (A, SK3530 drug substance solution; B, standard solution; C, heat stress (API solution); D, heat stress (API solid); E, oxidation condition; F, acidic condition; G, alkaline condition).

products obtained with on-line DAD detection. The UV spectrum of degradation compound 3 was found quite similar to the spectrum of SK3530.

Thus, the developed assay method in this study is stability-indicating since it enables the separation of SK3530 from its related compounds and degradation products as well as quantitative determination of SK3530 in the presence of other compounds.

4.2. Linearity

Linearity of the HPLC method was evaluated over the SK3530 concentration range of 2.51–50.11 and 200–300.7 μg/ml at pure standard solution as well as in the drug-matrix solution. This range corresponds to 1–20% and 80–120% of the intended test concentration of 250 μg/ml for pharmaceutical quality control of API and the drug in its tablet. Table 2 shows that the correlation coefficients were better than 0.999 in most cases. Furthermore, the linearity of the calibration curves for the compounds related to SK3530 were also studied over the ranges between 0.125 and 3.0 μg/ml. This range corresponds to 0.05–1.20% of intended test concentration of 250 μg/ml for pharmaceutical quality control of SK3530 API and the drug in its tablet. Table 2 also shows that the correlation coefficients were better than 0.9999 for all the cases in either pure solutions of in solutions comprising the drug-matrix. The *y*-intercept% was calculated target concentration response at assay or 1.2 wt.% impurity response at impurity, respectively. The *y*-intercept% not more than 2.0% found for all three calibration curves.

4.3. Accuracy and precision

The results of accuracy were expressed as percent of recovery of the particular components from the sample solutions. The accuracy results were expressed as percent recoveries of the particular components in the samples. The overall percent of recoveries of SK3530 in pure and drug-matrix solutions were 100.0 (relative standard deviation (R.S.D. = 0.34%) and 100.0 (R.S.D. = 0.26%), respectively. However, the related compounds showed the overall percent of recoveries ranging from 100.1 to 101.0 with R.S.D.s ranging from 1.14 to 3.25%.

The first type of precision, as 10 injections of 0.05% and 100% sample solution, was 5.6% and 0.2% at 0.05% and 100% sample, respectively. The result of precision for within-run and between-run was listed in Table 3. Table 3 shows that

Table 2
Linearity of calibration curves for SK3530 and its related compounds in standard preparations and in the drug-matrix preparation. Number of points in the regression is 5 for SK3530 and 7 for its related compound

Compound	Calibration range (μg/ml)	Correlation coefficient	Slope	95% confidence intervals of the slope[a]	Intercept	95% confidence intervals of the intercept[a]	LOD (μg/ml)	LOQ (μg/ml)
Standard preparations								
SK3530	2.51–50.11	1.000	37.0	±0.192	0.859	±2.59	0.07	0.22
SK3530	200–300.7	0.9996	36.2	±0.473	118	±98.5	2.82	8.55
Impurity A	0.116–2.791	1.000	36.8	±0.0598	0.0482	±0.274	0.01	0.02
Impurity B	0.123–2.962	1.000	41.0	±0.0532	−0.167	±0.192	0.00	0.01
Impurity C	0.119–2.851	1.000	45.4	±0.706	−0.448	±0.524	0.01	0.04
Impurity D	0.124–2.969	0.9999	37.0	±0.530	−0.523	±1.21	0.03	0.10
Drug-matirx preparations								
SK3530	2.51–50.11	1.000	37.1	±1.48	0.448	±4.74	0.13	0.40
SK3530	200–300.7	0.9999	36.3	±1.15	−11.6	±113	3.24	9.81
Impurity A	0.116–2.791	1.000	36.1	±2.92	0.916	±1.95	0.06	0.17
Impurity B	0.123–2.962	1.000	40.3	±0.890	−0.0973	±0.201	0.01	0.02
Impurity C	0.119–2.851	1.000	48.5	±3.66	−0.492	±0.909	0.02	0.06
Impurity D	0.124–2.969	1.000	36.4	±1.04	−0.485	±0.572	0.02	0.05

[a] Confidence intervals of the slope and the intercept = S.D. of the slope or intercept × *t*; the value of *t* at 3 degrees of freedom and 95% confidence level is 3.18.

Table 3
Results of SK3530 quality control samples

Concentration (nominal, μg/ml)	Within-run precision (%)	Between-run precision (%)	Accuracy (%)
203.3	0.098	0.12	100.3
254.2	0.059	0.18	100.0
305.0	0.060	0.17	99.89

$n = 3$ per concentration level.

within-run and between-run precisions were not less than 0.2% with accuracies between 99.9 and 100.3%.

4.4. LOD and LOQ

The values for LOD and LOQ are given in Table 2. The chromatogram with SK3530 of 0.125 μg/ml (0.05% of the label claim) showed signal to noise ratio approximately as 11/1 and exhibited 5.6% (%R.S.D.) of value for 10 times repeatability.

4.5. Stability of sample solutions

Stability for sample solutions of SK3530 and SK3530 with tablet excipients was checked by analyzing their solutions aged over 3 days. The percent difference observed between initial and value after 3 days was in the range of −0.83 to 0.60%, indicating that there are no additional peaks nonetheless using standard solutions of SK3530 in pure or drug-matrix solutions lapsed a period of 3 days.

4.6. System suitability parameters

The observed linearity of the analytical method supports the use of a single standard concentration for the standardized quantitative analysis of SK3530. The system suitability will consist of 1 blank injection (mobile phase), 10 injection of the SK3530 standard reference solution and 1 injections of a separately weighed SK3530 standard reference solution. Criteria for retention factor, theoretical plate, tailing factor, %R.S.D. in area (10 injections), and ratio between the area of the separately weighed standard reference solutions were defined as: $k' \geq 4$, $N > 6000$, $0.5 \leq T \leq 2$, %R.S.D. < 1.0%, ratio 0.99–1.01, respectively.

The SK3530 content of sample solutions will be calculated using the bracket mode, in which the area of two preceding and two following standard reference solutions are used.

5. Conclusions

An HPLC method for the assay of SK3530 and its related compounds in the API and its tablet formulation was validated in this study. SK3530 and the other related compounds which may coexist with it as impurities or as degradants gave chromatograms of very well resolved peak which indicate the specificity of the method and the possibility of using it as an indicator of stability. All the statistical values (percent of recoveries, R.S.D., confidence intervals of the slope and the intercept, LOD and LOQ) calculated were within the acceptable limits.

The validation data provided here indicated that the chromatographic assay for SK3530 tablets is transferable method and is suitable for regulatory filing. The method satisfies the regulatory requirements of linearity, precision and selectivity to quantitate SK3530 in the tablets.

The developed stability-indicating LC-UV method will be used for the pharmaceutical quality control of SK3530 API and its tablet.

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