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Simultaneous determination of ampicillin sodium and sulbactam sodium in powder for injection by HPLC

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Abstract Ampicillin and Sulbactam (2:1, w/w) are combined in formulation to provide broader antibacterial action in treatment of many infections. The development of analytical method for simultaneouly determine these two compounds was difficult because of the differences in their chemical structures and ratio in the formulation. Current published methods still have some limitations. In this study, we developed an alternative high-performance liquid chromatography (HPLC) assay method for simultaneously determination of ampicillin sodium and sulbactam sodium in powder for injection. Method validation of HPLC method was conducted to determine linearity, precision, accuracy, system suitability, robustness. The linearity of the calibration curves in the desired concentration range was good ($r^2 > 0.9994$). RSDs of intra-day and inter-day precision obtained were less than 2.00 %. Accuracy was obtained with the recoveries in range of 98.42 % and 101.36 %. As a result of system suitability, RSD of both retention time and the peak area obtained were not more than 1.0 %. The values of plate number were more than 7000 and symmetric factors obtained were 0.8. As intermediate-precision and robustness of the developed assay, it could be expected to become valuable tools for revising the Korean Pharmacopoeia (KP XI).

Key words: high-performance liquid chromatography (HPLC), Korean Pharmacopoeia (KP XI), ampicillin sodium, sulbactam sodium, assay

1. Introduction

Ampicillin sodium (*Fig.* 1(a)) is chemically named as sodium (2S,5R,6R)-6-[[(2R)-2-amino-2-phenylacetyl] amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0] heptane-2-carboxylate. Ampicillin (AMP) is a semi-synthetic derivative of aminopenicillin which inhibits bacterial cell wall synthesis by inhibiting peptidoglycan synthesis, a critical component of the bacterial cell wall.[1] Ampicillin has a broad spectrum of bactericidal

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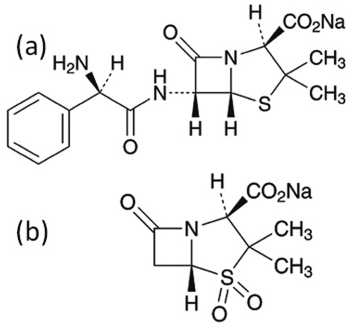

Fig. 1. Chemical structures of (a) ampicillin sodium and (b) sulbactam sodium.

activity against many gram-positive and gram-negative aerobic and anaerobic bacteria. Ampicillin is, however, degraded by beta-lactamases and therefore the spectrum of activity does not normally include organisms which produce these enzymes.[2] Sulbactam sodium (*Fig.* 1(b)) is chemically named as sodium (2S,5R)-3,3-dimethyl-4,4,7-trioxo-4λ^6-thia-1-azabicyclo[3.2.0] heptane-2-carboxylate. It is a beta-lactamase inhibitor with very weak antibacterial action. Sulbactam sodium (SUL) contains a beta-lactam ring and irreversibly binds to beta-lactamase at or near its active site, thereby blocking enzyme activity and preventing metabolism of other beta-lactam antibiotics.[3] Combining this agent with a beta-lactamase sensitive antibiotic such as penicillins and cephalosporins against penicillinase-producing and beta-lactamase-producing organisms results in a decreased turnover rate of the sensitive antibiotic and enhances its antibacterial property by 4- to 32-fold when compared to ampicillin alone.[4,5]

AMP and SUL are formulated as powder for injections (AMP: SUL = 2:1 w/w) in treatment of many infections. Although a suitable analytical method is very crucial for drug quality control, the difference in these two compounds' chemical structures as well as the doubled amount of AMP in the formulation lead to difficulties of simultaneous determination of AMP and SUL. From the review of literature, it was found that high-performance liquid chromatography (HPLC) methods are available for the determination of AMP, individually and in combination with other related compounds.[6-8] To investigate the pharmacokinetics of AMP/SUL (2:1) combination after intravenous administration, concentrations of AMP and SUL were determined separately using two different analytical methods or applying post-column reaction technique which is complicated for routine quality control purposes.[9,10] Currently, in Korean Pharmacopoeia XI (KP XI), United States Pharmacopoeia 41 (USP 41), Japanese Pharmacopoeia XVII (JP XVII) HPLC methods were used for the assay test of AMP/SUL (2:1) powder for injections.[11-13] However, these method still have some disadvantages when being applied in routine drug quality control such as (1) high back pressure, which reduce the lifetime of analytical column, (2) abnormal tailing and broadening peak shapes, (3) requiring large amount of organic solvents, (4) long system stabilization time.

Therefore, the objective of the present work is to develop a new HPLC method for simultaneously quantitation of AMP and SUL in powder for injection that can be substituted the present assay tests. Validation was conducted following the International Conference on Harmonization (ICH) and Korean Food and Drug Administration (KFDA) Validation Protocols.[14-16]

2. Experimental

2.1. Chemicals and reagents

Sulbactam sodium (≥ 99.0 %) and ampicillin sodium (≥ 95.0 %) standard were purchased from Sigma-Aldrich (St. Louis, USA). Sulbactam and ampicillin injection was supplied by Shinpoong Pharm.co., LTd (Ansan, Korea). Potassium phosphate monobasic (≥ 99.0 %), HPLC grade acetonitrile (99.9 %) and methanol (99.9 %) were obtained from Daejung Chemicals and Metals Co. (Siheung, Korea). Purified water was prepared in our laboratory. All other chemicals were of analytical reagent grade.

2.2. Instrumental conditions

Experiments were conducted on Agilent 1100 HPLC system included G1379A Degasser, G1312 Binary Pump, G1313 Auto-sampler, G1316 Colcom (Column Oven) and G1314AVWD Detector (Agilent Technology,

Santa Clara, USA). In intermediate precision validation, Shimadzu HPLC system consisted of following components: DGU – 20A5R Degasser, two LC – 20 AD pumps, SIL – 20A autosampler, SPD-20A UV – Vis Detector, CBM – 20A communication bus module (Shimadzu Corporation, Kyoto, Japan) and CO-965 Column Oven (Jasco Corporation, Tokyo, Japan) was used.

For the HPLC condition, a Aegispak C18-F column (150 × 4.6 mm I.D., 5 μm) was used for for gradient elution by using a binary mixture of eluent A (10 mM potassium dihydrogen phosphate at pH 2.8 and methanol (85:15, v/v)) and eluent B (10 mM potassium dihydrogen phosphate at pH 2.8 and methanol (40:60, v/v)) at a flow rate of 1.0 mL/min. Gradients started at 95 % of eluent A and 5 % of eluent B for 2.5 min and later increased to 55 % of eluent B over 1 min. This ratio was then maintained for 2 min. Then, the ratio of eluent B was decreased linearly to 5 % over 1 min. Finally, this process finished with an isocratic elution step for 5 min. A detector wavelength of 225 nm was used to quantify both SUL and AMP. Injection volume was 20 μL.

2.3. Sample preparation

Stock solutions of SUL and AMP were prepared by dissolving 50 mg of SUL and 100 mg of AMP in 50 mL water to obtain concentrations of 1000 μg/mL and 2000 μg/mL, respectively. To prepare standard solution, appropriate volumes of the above stock solutions were taken and diluted with water to obtain a concentration of 250 μg/mL SUL and 500 μg/mL AMP.

Sample solution: The content of a vial of AMP and SUL for injection was mixed. 75.0 mg of the mixture was accurately weighed and dissolved in 25 mL water. This solution was quantitatively diluted to obtain a solution of 250 μg/mL SUL and 500 μg/mL AMP. A portion of this solution was passed through a 0.45 μm membrane filter as the sample solution.

2.4. Validation studies

Method was validated accordingly to ICH Q2 (R1) guideline with regard to linearity, precision, accuracy and robustness.

The linearity of the proposed HPLC procedure was evaluated using different concentrations (20 – 400 %) of the analytes (250 μg/mL for SUL and 500 μg/mL for AMP). Linearity was estimated by coefficient of determination (r^2) of the regression lines from 6 repeated analyses of the desired concentration range. Precision (relative standard deviation, RSD %) of the method were assessed by six analyses in a day (Intra – day) and in three different days (Inter – day) of standard solutions at concentrations corresponding to 80, 100, 120 % of analysis concentration (200; 250; 300 for SUL and 400; 500 and 600 μg/mL for AMP). Accuracy was expressed as recovery rates that were evaluated using the standard addition method: three concentrations (200; 250; 300 μg/mL for SUL and 400; 500; 600 μg/mL for AMP) were added into a sample solution consisting of 250 μg/mL of SUL and 500 μg/mL of AMP. The experiments were performed in triplicate.

2.5. Application of the method

This analytical method was applied to quantitate the content of SUL and AMP in powder for injection. The study was conducted on 6 samples prepared from powder as mentioned above. The amount of SUL and AMP in sample was calculated by following expression:

Sulbactam sodium ($C_8H_{11}NO_5SNa$) (mg) = m_{SUL} x ($A_{T(SUL)}$ / $A_{S(SUL)}$)

Ampicillin sodium ($C_{16}H_{19}N_3O_4SNa$) (mg) = m_{AMP} x ($A_{T(AMP)}$ / $A_{S(AMP)}$)

Where

m_{SUL}, m_{AMP} (mg) are the amount of SUL and AMP weighed,

$A_{S(SUL)}$, $A_{S(AMP)}$ (mAU*s) are peak area of SUL and AMP in standard solution,

$A_{T(SUL)}$, $A_{T(AMP)}$ (mAU*s) are peak area of SUL and AMP in sample solution.

3. Results and Discussion

3.1. Investigation of compendial methods

Following JP XVII monograph of ampicillin sodium and sulbactam sodium for injection,[13] a C18 column

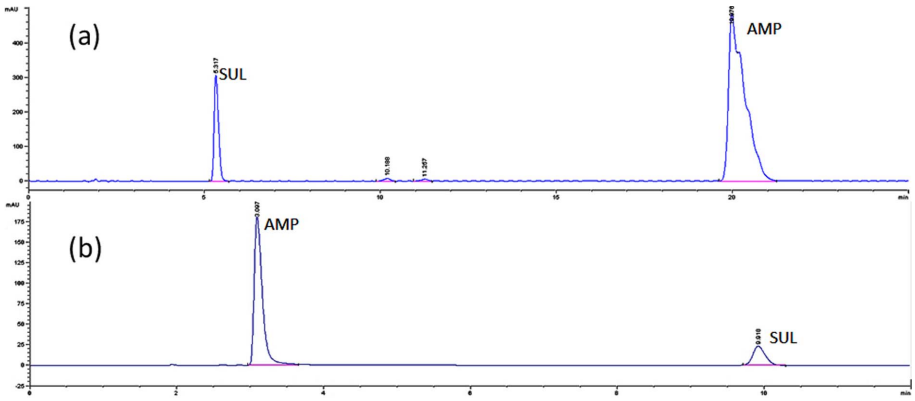

Fig. 2. Typical chromatograms of (a) JP XVII method: Aegispak C18-F column (150 × 4.6 mm I.D., 5 µm) thermostated at 35 °C with mobile phase included mixture of 0.02M phosphate buffer (pH 3.0) and acetonitrile (23:2, v/v); flow rate: 1.0 mL/min; injection volume: 10 µL; UV Detection at 215 nm. (b) USP 41/KP XI method: Aegispak C18-L column (250 × 4.6 mm I.D., 5 µm) with mobile phase included mixture of 0.005M Tetrabutylammonium hydroxide (pH 5.0) and acetonitrile (1650:350, v/v)); flow rate: 2.0 mL/min; injection volume: 10 µL; UV Detection at 230 nm.

(150 × 4.0 mm I.D., 5 µm) thermostated at 35 °C was used for the analysis of AMP and SUL. Mobile phase included mixture of 0.02M phosphate buffer (pH 3.0) and acetonitrile (23:2, v/v). As can be seen in *Fig.* 2(a), chromatogram obtained shows tailing and splitting peak of AMP. The running time was also more than 20 min. Thus, it is difficult to apply this condition in routine analysis.

In KP XI[11] and USP 41,[12] C18 column (300 × 4.0 mm I.D., 5 µm) was used for the analysis of AMP and SUL. Mobile phase included mixture of 0.005M Tetrabutylammonium hydroxide (pH 5.0) and acetonitrile (1650:350, v/v). This chromatographic condition shows improvement in running time and peak shape in compared with JP method (*Fig.* 2(b)). However, due to high flow rate (2.0 mL/min), the method causes a high back pressure on analytical column (250 bar) that might decrease its lifetime and consume large amount of organic solvent and chemical reagents. Therefore, we aimed to develop an improve method for the analysis of the two compounds using 150 mm length column and mobile phase include methanol (MeOH) or acetonitrile (ACN) and potassium dihydrogen phosphate solution.

3.2. Development of the HPLC method

In preliminary experiments, the effect of concentration of methanol (from 5 to 30%) and pH of phosphate buffer (from 2.8 to 3.6) were investigated. AMP, which is relatively nonpolar, was not eluted within 30 min until the concentration of methanol was increased to 20 % in mobile phase. However, at that ratio, SUL, which is more polar, was not sufficient retained in the column (capacity factor (k') = 1.2, number of theoretical phate (N) was about 4000). Additionally, even when the concentration of methanol was increased to 30%, AMP's peak shape was severely tailing. pH of phosphate buffer also has important impact on chromatographic parameters of the two analytes. With an increased pH, the retention of SUL and AMP were both reduced; symmetry and N of SUL peak were dereased and those of AMP were increased (*Table* 1).

The different retention properties of SUL and AMP inquired to apply gradient profiles instead of an isocratic condition. Trials were carried out, during which, the composition of buffer and methanol at a fixed flow rate of 1.0 mL/min was changed (*Supplemental material*). Among trials performed, a gradient program was finalized, in which both components were well separated (*Table* 2).

The optimized HPLC condition were: a Aegispak C18-F column (150 × 4.6 mm I.D., 5 µm) was used for for gradient elution by using a binary mixture of eluent A (10 mM potassium dihydrogen phosphate at pH 2.8 and methanol (85:15, v/v)) and eluent B

Table 1. Investigation of mobile phase

Factor	Value	SUL				AMP			
		t_R (min)	k'	N	Sym	t_R (min)	k'	N	Sym
% MeOH (%) (With 10 mM phosphate buffer pH 3.0)	5	12.8	6.6	8314	0.49	>60	-	-	-
	10	7.0	3.1	5344	0.35	>60	-	-	-
	15	4.8	1.8	5543	0.50	>45	-	-	-
	20	3.8	1.2	5076	0.62	17.2	9.1	3652	0.21
	25	3.2	0.9	4040	0.66	10.2	5.0	4251	0.29
	30	2.8	0.7	3612	0.68	6.6	2.9	4854	0.40
pH of buffer (with 25% of MeOH)	2.8	4.0	1.4	5336	0.75	14.3	7.4	1687	0.23
	3.0	3.2	0.9	4040	0.66	10.2	5.0	4251	0.29
	3.2	3.1	0.9	4503	0.64	9.2	4.4	7037	0.52
	3.4	2.9	0.7	3316	0.56	9.0	4.3	7874	1.11
	3.6	2.8	0.7	2642	0.49	8.7	4.1	6526	1.99

t_R (min): retention time
k': capacity factor
N: number of theoretical plate
Sym: Symmetric factor

Table 2. Gradient program

Time (min)	Eluent A (%)	Eluent B (%)
0.0 – 2.5	95	5
2.5 – 3.5	95 → 45	5 → 55
3.5 – 5.5	45	55
5.5 – 6.5	45 → 95	55 → 5
6.5 – 10	95	5

Eluent A: 10 mM potassium dihydrogen phosphate pH 2.8 : MeOH = 85 : 15 (v/v)
Eluent B: 10 mM potassium dihydrogen phosphate pH 2.8 : MeOH = 40 : 60 (v/v)

(10 mM potassium dihydrogen phosphate at pH 2.8 and methanol (40:60, v/v)) at a flow rate of 1.0 mL/min; a detector wavelength of 225 nm was used to quantify both SUL and AMP. Injection volume was 20 μL. Typical chromatogram was shown in *Fig.* 3(b).

In comparison with published methods, the peak heights and shapes obtained from optimized condition were improved, result in more accurate measurement of peak areas and enhance the sensitivity of analytical method. The back pressure of HPLC column was

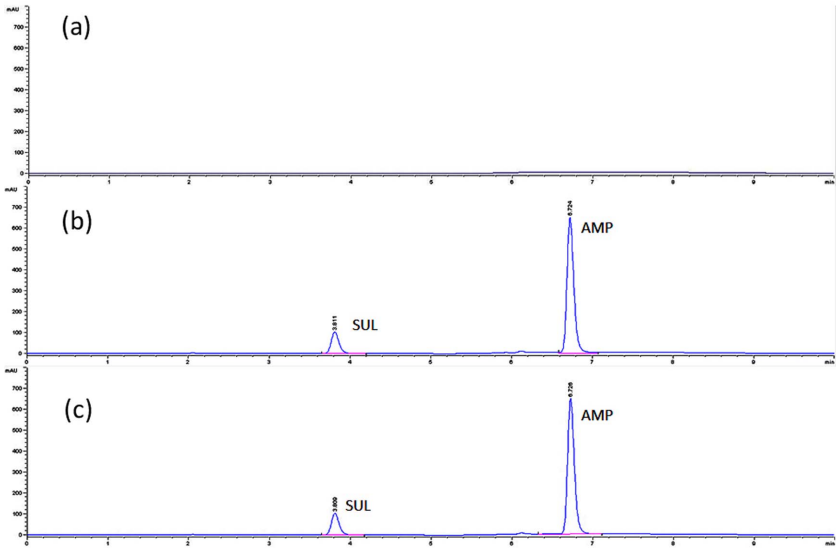

Fig. 3. Typical chromatograms of (a) blank water sample (b) 250 μg/mL of SUL and 500 μg/mL of AMP standard solution (c) sample solution prepared from powder for injection. Condition: Aegispak C18-F column (150 × 4.6 mm I.D., 5 μm) was used for gradient elution at a flow rate of 1.0 mL/min; injection volume: 10 μL; UV Detection at 225 nm.

Table 3. Comparison between the developed method and USP41/KP XI method (n = 6)

Comparison			Developed method	USP 41/ KP XI
Condition	Stationary phase		C18 (150 × 4.6 mm, 5 μm)	C18 (250 × 4.6 mm, 5 μm)
	Mobile phase		Gradient Elution Eluent A & B (*Table* 2)	Isocratic Elution TBA : ACN = 165 : 35
	Flow rate (mL/min)		1.0	2.0
	Column pressure (bar)		104	250
Chromatographic parameters	SUL	t_R (min)	3.81	9.71
		k'	1.3	10.5
		N	7969	16779
		Height (mAU)	102.9	32.1
		Sym	0.80	0.75
		RSD of t_R (%)	0.19	2.17
		RSD of area (%)	0.24	1.93
	AMP	t_R (min)	6.73	3.22
		k'	3.0	2.8
		N	32928	4511
		Height (mAU)	649.6	159.2
		Sym	0.76	0.51
		RSD of t_R (%)	0.08	1.85
		RSD of area (%)	0.99	1.22

TBA: 0.005M Tetrabutylammonium hydroxide (pH 5.0)
ACN: Acetonitrile
Eluent A: 10 mM potassium dihydrogen phosphate pH 2.8 : MeOH = 85 : 15 (v/v)
Eluent B: 10 mM potassium dihydrogen phosphate pH 2.8 : MeOH = 40 : 60 (v/v)
RSD (%): Relative standard deviation

also reduced from more than 200 bar to about 100 bar while running time was maintained less than 10 minutes. Other chromatographic parameters demonstrated the improvement of the method compared to USP 41/ KP XI are shown on the comparison table (*Table* 3).

3.3. Method validation

3.3.1. Linearity

Calibration curves showed good linearity in the concentration range 50 ~ 1000 μg/mL for SUL and 100 ~ 2000 μg/mL for AMP (*Table* 4). The coefficient of determination was 1.0000 for SUL and 0.9994 for AMP, which indicates a good correlation between the peak areas and the range of concentrations studied. The LOD and LOQ concentrations of SUL were estimated to be 3.0 and 10.0 μg/mL, respectively while those of AMP were 0.5 and 1.0 μg/mL when signal-to-noise ratios of 3 and 10 were used as the criteria.

Table 4. Results of linearity validation

Parameter	SUL	AMP
Regression equation	y = 2.6677x + 5.7689	y = 6.9108 x + 377.05
Range (μg/mL)	50 – 1000	100 – 2000
Correlation coefficient (r^2)	1.0000	0.9995
Number of data points	5	5
Slope ± SD	2.6677 ± 0.01	6.9108 ± 0.08
Intercept ± SD	5.7689 ± 2.76	377.05 ± 30.26

SD: Standard deviation

3.3.2. Precision

The precision of the method was assessed by determining the intra-day assay relative standard deviation (RSD %) of the analysis (n = 6) of standard solutions at three concentrations: 200; 250; 300 μg/mL of SUL and 400; 500 and 600 μg/mL of AMP). Three replicates of each concentration were analyzed on each of three consecutive days. Results obtained are shown in *Table* 5. The intra-day precision for each concentration was 0.91 ~ 1.89 % and the inter-

Table 5. Results of precision (intra/inter-day) validations of the proposed method

Compound	Conc. (μg/mL)	Intra-day (n = 6)		Inter-day (n = 12)	
		RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
SUL	200	0.91	100.92	1.95	98.91
	250	1.36	98.27	1.11	98.79
	300	1.83	98.50	1.58	98.99
AMP	400	0.93	101.65	1.92	101.70
	500	1.89	101.37	1.58	102.09
	600	1.52	101.21	1.42	102.42

Table 6. Recovery tests for SUL and AMP for powder for injections (n = 3)

Added conc. (%)	SUL		AMP	
	Mean (%)	RSD (%)	Mean (%)	RSD (%)
80	99.17	1.85	100.10	1.44
100	99.69	1.14	100.21	1.05
120	98.42	1.04	101.36	1.39

Table 7. System suitability data (n = 6)

Compound	Retention time (RSD %)	Peak area (RSD %)	Plate number	Symmetric factor
SUL	0.19	0.24	7969	0.80
AMP	0.08	0.99	32928	0.76

day precision was 1.11 ~ 1.92 % for both analytes.

3.3.3. Recovery

Results of recovery studies by standard addition method were ranged from 98.42 % to 99.69 % for SUL and 100.10 % to 101.36 % for AMP (*Table* 6). This also suggested that there was no interference from excipients in determining content of SUL and AMP in injections.

3.3.4. System suitability, robustness and intermediate precision

Relative standard deviations of retention time, peak areas and number of theoretical plates, symmetric factor was measured after 6 repeats of 250 μg/mL SUL and 500 μg/mL AMP solution analyses to evaluate system suitability of method (*Table* 7). RSD % of retention time and peak areas were less than 1.0 %. The average numbers of theoretical plates were 7969 for SUL and 32928 for AMP. Average symmetric factor was 0.8 for both compounds.

Table 8. Contents of SUL and AMP in powder for injections (n = 6)

Compound	Claimed value	Assay	
		Content (%)	RSD (%)
SUL	250 mg	97.99	1.68
AMP	500 mg	101.17	0.75

Robustness of the method was checked by making small deliberate changes in the buffer pH (2.8 ± 0.1) and column temperature (25 ± 1 °C). In both cases, except changes in retention time, the results of method were not affected: RSD % of peak area (n = 6) was not more than 2.0 %, number of theoretical plates were more than 7000 for SUL and more than 30000 for AMP and symmetric factor was not less than 0.75 and not more than 0.9.

Intermediate precision was studied by using Shimadzu HPLC system. Results showed that even though there was an increase in retention time of AMP - about 1.5 minutes later compared to Agilent system, it did not affect to the precision of this method. Tailing factor were 1.26 and 1.35 for SUL and AMP, respectively. The number of theoretical plates was about 5741 for SUL and 44369 for AMP. RSD% of peak area was less than 0.50 % for both compounds.

3.6. Application

This analytical method was applied to simultaneously quantitate the content of SUL and AMP in injections. The results of assay test in 6 samples of commercial injections were recorded in *Table* 8. The average content of SUL in the formulation was 97.99 % (RSD = 1.68 %), that of AMP was 101.17 % (RSD

= 0.75 %). A typical chromatogram of sample is shown in *Fig*. 3(c).

4. Conclusions

Althought AMP and SUL combination have been administered for a long time in clinical treatment, the current assay methods pulished still have some limitations (instrumental operation and management, amount of organic solvent consume, chromatographic parameters). By using the developed gradient elution, AMP and SUL (2:1) combination can be quantitated rapidly and precisely despite their remarkable differeces in properties and concentrations. Through the validation results, the proposed method is demonstrated worth replacing previous tests and employing in quality controls of formulations that contain these two compounds.

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