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A Validated Stability Indicating RP-LC Method for the Determination of Related Impurities in S-Adenosyl-L-Methionine (SAMe) API

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ABSTRACT

A stability indicating gradient reversed-phase liquid chromatographic (RP-LC) related substance method for S-Adenosyl-L-methionine (SAMe) in active pharmaceutical ingredient was developed and validated. SAMe was subjected to stress conditions and observed sensitive to alkali hydrolysis, oxidation and thermal degradation. Successful separation of SAMe from its process and degradation related impurities was achieved on a YMC-Pack Pro-C18, 150 mm × 4.6 mm, 3µm column using a gradient mixture of solvent A (mixture of 0.015 M citric acid monohydrate, 0.01 M sodium dihydrogen orthophosphate dihydrate, 0.014 M sodium lauryl sulphate and acetonitrile in the ratio of 75:25 v/v) and solvent B (mixture of 0.004 M citric acid monohydrate, 0.003 M sodium dihydrogen orthophosphate dihydrate, 0.014 M sodium lauryl sulphate and acetonitrile in the ratio of 20:80 v/v). The flow rate was 1.5 mL/min and the detection wavelength was 254 nm. The mass balance was found to be in the range of 99.1 - 99.9% in all the stressed conditions.

Keywords: S-Adenosyl-L-methionine (SAMe), HPLC, Degradants, Stability indicating method

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INTRODUCTION

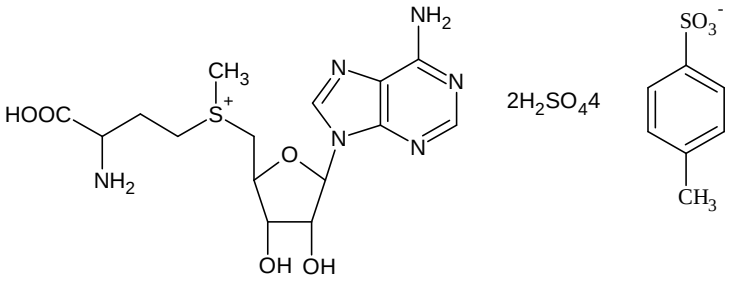
S-Adenosyl-L-methionine (SAMe) chemically known as 5'-[(3-amino-3-carboxy-propyl) methylsulfonio]-5'-deoxy adenosine hydroxide (Figure.1) is a naturally occurring substance present in plasma and most tissues. It is commercially available and marketed worldwide as a nutritional supplement that is readily available in vitamin and health food stores. It is the principal methyl donor within the body for trans methylation reactions. This is also a vital precursor for the trans-sulfuration pathway. The final product of the trans-sulfuration pathway is glutathione (GSH). SAMe is metabolized to S-Adenosylhomocysteine (SAH) and then to homocysteine by a hydrolase enzyme. Homocysteine is converted to cystathionine by cystathione β–synthase and cystathionine is subsequently converted to cysteine, a precursor for GSH, by γ-cystathionase. Cysteine is subsequently converted by glutamate-cysteine lagase, the rate limiting enzyme in GSH synthesis to γ–glutamylcysteine, which is finally converted by GSH synthetase to GSH [1-4].

Therapeutic use of SAMe has increased word wide, as dietary supplements have gained in popularity, especially after the Dietary Supplement Health and Education Act was passed in 1994. This law allowed the distribution of SAMe as a dietary supplement, and therefore allowed it to bypass the regulatory requirements for drugs of the Food and Drug Administration (FDA).

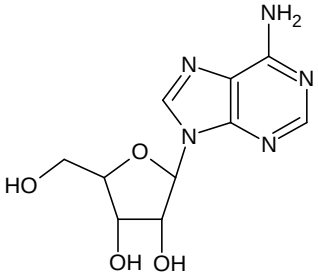
Few HPLC methods were reported in the literature for the quantitative determination of S-Adenosyl-L-Methionine (SAMe) in Assay [4, 5]. A comparative study of the reversed-phase HPLC retention behaviour of S-Adenosyl-L-Methionine (SAMe) and its related metabolites on different stationary phases were studied[6]. Also HPLC analysis of SAMe in pharmaceutical formulations were reported earlier[7].

So far, to our present knowledge, no stability indicating related substances analytical method for S-Adenosyl-L-Methionine (SAMe) was available in literature. It was felt necessary to develop a stability indicating LC method[8] for the related substance determination and quantitative estimation of S-Adenosyl-L-Methionine(SAMe). This paper describes the related substance method validation[9] for accurate quantification of S-Adenosyl-L-Methionine (SAMe) and all six impurities in bulk drug samples, respectively.

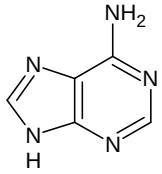
Figure 1. Chemical Structures of SAMe and Impurities A, B,C, D, E and F



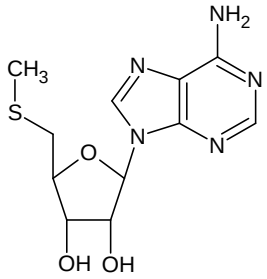
(3S)-5'-[(3-Amino-3-carboxypropyl)methylsulfonio]-5'-deoxyadenosine hydroxide, disulphate, methanebenzenesulfonate (S-Adenosyl-L-Methionine disulphate p-toluene sulphonate (SAMe))



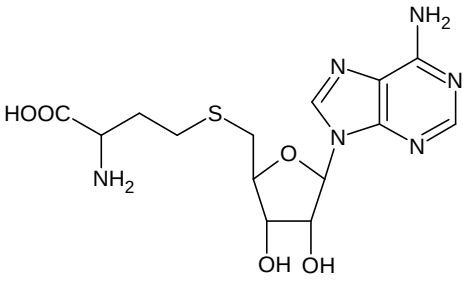
9-β–Ribofuranosyl-9H-purine-6-amine (Adenosine) **Impurity-A**



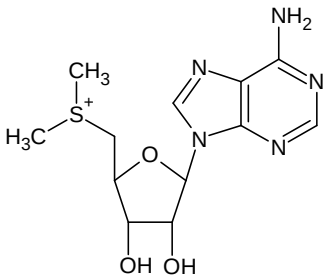
6-Aminopurine (Adenine) **Impurity-B**



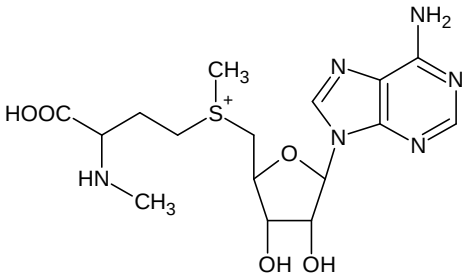
5'-(Sulfonylmethane)-5'-deoxyadenosinehydroxide (Methylthio adenosine) **Impurity-C**



5'-[(3-Amino-3-carboxy-propyl)methanesulfanyl] 5'-deoxyadenosinehydroxide (Adenosine Homo Cysteine) **Impurity-D**



5'-Deoxyadenosine hydroxide-5'-dimethyl sulfonium **Impurity-E**



5'-[3-Aminomethyl-3-carboxy-propyl)methylsulfonio] -5'deoxyadenosine hydroxide **Impurity-F**

MATERIALS AND METHODS

Chemicals:

Active pharmaceutical ingredient of SAMe and its process related and degradation impurities **(**Figure 1**)** were received from process research department of Orchid chemicals and pharmaceuticals limited, Chennai, India. HPLC grade acetonitrile, analytical reagent grade sodium dihydrogen orthophosphate dihydrate, citric acid mono hydrate

and electrophoresis grade of sodium lauryl sulphate were purchased from Merck, Mumbai, India. All the solutions were prepared in Milli Q water (Millipore, USA).

HPLC instrumentation and conditions:
Waters Alliance 2695 separation module (Waters Corporation, Milford, USA) equipped with 2487 UV/visible detector or 2996 PDA detector (for specificity and forced degradation studies) with Empower 2 software was used for the analysis. YMC-Pack Pro- C18 (150 mm × 4.6 mm, 3µm, YMC Europe, Gmbh) and gradient mixture of solvent A and solvent B were used as stationary and mobile phases, respectively. The solvent A contains a mixture of 0.015 M citric acid mono hydrate, 0.01 M sodium dihydrogen ortho phosphate dihydrate, 0.014 M sodium lauryl sulphate, acetonitrile in the ratio 75:25 v/v. The solvent B contains a mixture of 0.004 M citric acid monohydrate, 0.003 M sodium dihydrogen orthophosphate dihydrate and 0.014 M sodium lauryl sulphate, acetonitrile in the ratio 20: 80 v/v. The gradient program was set as (time/% solution B): 0/5, 10/20, 20/40, 25/45, 30/70, 35/70, 36/5 and 45/5 with a post run time of 10 min. 1.5 mL/min flow rate and 20 µL injection volume were maintained. The detection was monitored at a wavelength of 254 nm.

Preparation of standard and sample solutions:
0.01 mg/mL solution of SAMe standard and 1.0 mg/mL solution of sample were prepared using solvent A as diluent.

RESULTS AND DISCUSSION

Characterization of Impurities:
The process and degradation related impurities A-F were characterized using IR, NMR and LC-MS [10].

Method development and optimization:
The HPLC method was optimized so as to obtain a stability indicating method that can resolve process and degradation related impurities from SAMe. 0.015 M citric acid mono hydrate buffer was initially chosen as solvent A and acetonitrile as solvent B. There was no retention of main peak observed. Different trials were carried out and the retention of main peak was observed in the presence of ion pair reagent. Hence 0.014 M sodium lauryl sulphate was added in the solvent A. The main peak was well retained. Different pH trials were also carried out and the peak shape was good only at pH 3.0. In order to improve the peak shape and maintain pH about 3.0, 0.01 M sodium dihydrogen orthophosphate dihydrate buffer was added in to the solvent A. The separation of impurities B & C and also SAMe and impurity E was found critical. To achieve better separation of these impurities different acetonitrile ratio was employed in solvent A and in solvent B. Among different makes of C18 columns tried, YMC Pack Pro-C18, 150 mm x 4.6 mm, 3 µ column allowed a rapid resolution between impurities. In the optimized conditions the resolution between Impurity E and SAMe was found not less than 2.0 and plate count for each SAMe isomer peak observed not less than 20000.

The developed method was found to be specific for SAMe diastereoisomer (R,S), SAMe diastereoisomer (S,S) and its six impurities namely Impurity A, Impurity B, Impurity C, Impurity D, Impurity E and Impurity F**.**

Method Validation

Specificity
Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. Stress testing of the drug substance can help identify the likely degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used.

Forced degradation studies were performed on SAMe to provide an indication of the stability indicating property and specificity of the proposed method. The stress conditions employed for degradation study includes light (UV light~525 Watt hours/meter^2, Visible light~1.99 Million lux hours), heat (90˚C for 30 minutes), acid hydrolysis (Conc.HCl, @70°C for 2 hours), alkali hydrolysis (0.1N NaOH @ room temperature) and oxidation (30% H_2O_2 @ 30°C for 30 minutes). Photo diode array detection was also used as evidence of specificity of the method, and to evaluate homogeneity of the peak.

Summary of the forced degradation results are given in Table 1**.** Impurities A, B, C and D are found to be degradation impurities and E and F are process related impurities.

Table 1. Summary of Forced Degradation Results

Stress type	% Degradants formed							%Assay	Mass balance (%)
	Imp A	Imp B	Imp C	Imp D	Imp E	Imp F	Total		
Acid hydrolysis	0.00	2.1	2.0	0.3	0.1	0.00	2.0	97.1	99.1
Alkali hydrolysis	0.00	13.7	0.3	0.3	0.1	0.00	12.0	87.9	99.9
Oxidation	8.7	2.0	0.00	0.00	0.1	0.00	9.0	90.6	99.6
Thermal	0.00	7.8	0.8	0.4	0.3	0.1	3.0	96.3	99.3
UV	0.00	5.2	0.3	0.1	0.2	0.00	3.0	96.8	99.8
Visible	0.00	3.3	0.2	0.1	0.1	0.00	1.0	98.2	99.3

HPLC chromatograms spiked samples of all known impurities, degradation chromatograms are shown in Figures. 2-5.

Figure 2. Typical chromatogram of SAMe spiked with impurities

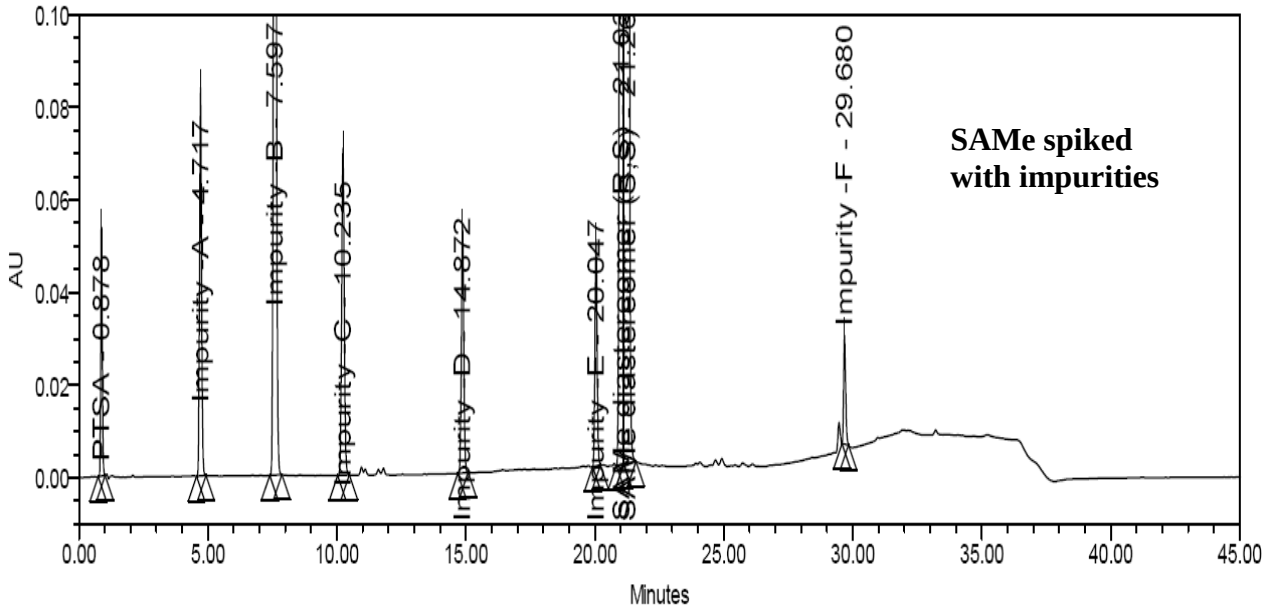


Figure 3. Typical Alkali degradation chromatogram.

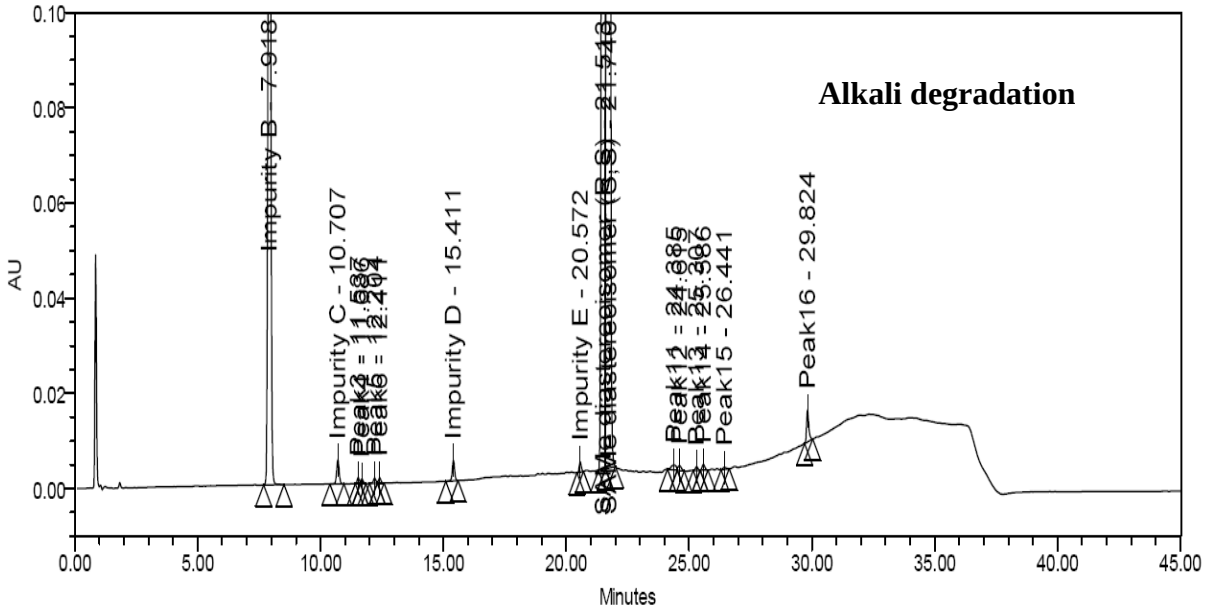


Figure 4. Typical Peroxide degradation chromatogram.

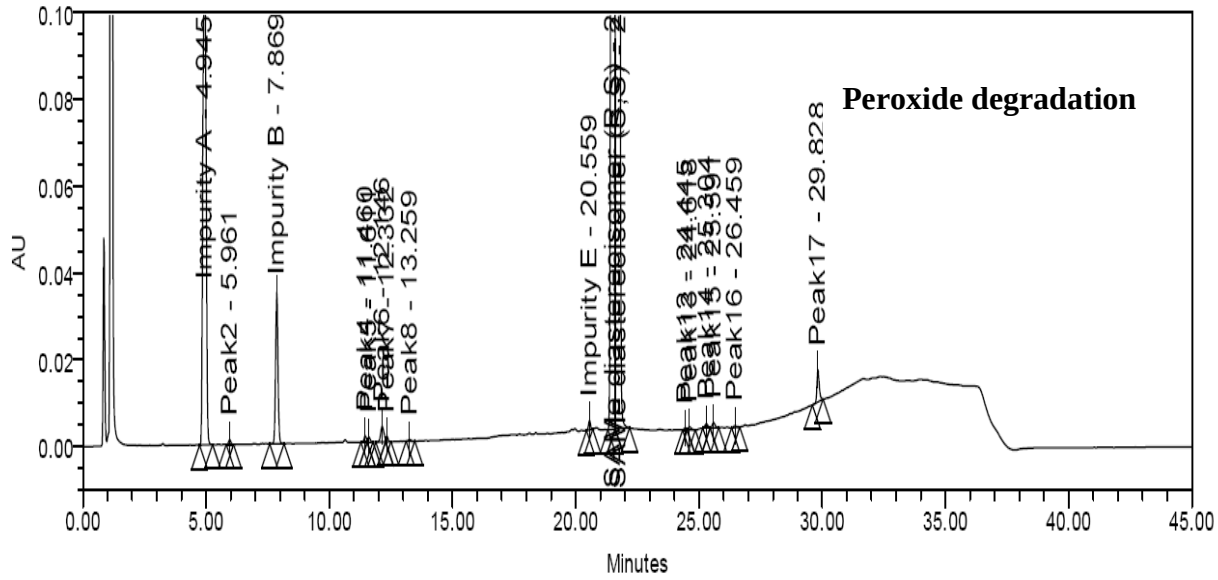
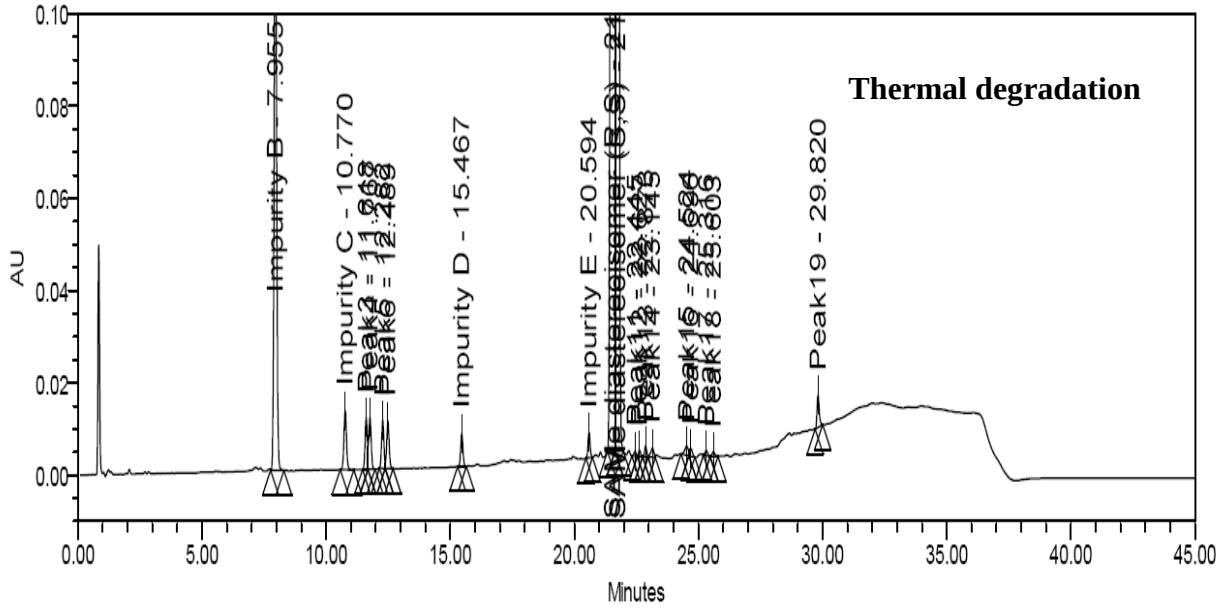


Figure 5. Typical Thermal degradation chromatogram.



Linearity:

Standard solutions at eight different concentration levels ranging from LOQ to 150 % of specification limit were prepared and analyzed in order to demonstrate the linearity for all the impurities. Linearity regression analysis demonstrated acceptability of the method for quantitative determination range of LOQ to 150 % of the specification limit. The correlation coefficient obtained was greater than 0.99. The slope and Y-intercept of the calibration curves shows that an excellent correlation existed between the peak area and concentration of all known impurities.

Accuracy:

Accuracy of the method was determined at five different concentration levels in triplicate. The analysis was carried out at LOQ, 50%, 75%, 100% and 150% of specification limit. The recoveries of all the impurities were found to be in the range of 80 – 120 % (Table 2).

Precision:

Repeatability was demonstrated by analyzing six individual preparations of SAMe spiked with 1.5% each of Impurities A, C, D, E, F and 3.0% of Impurity-B with respect to analyte concentration. The intermediate precision of the method was also evaluated using different analyst, instrument, column and in different day in the same

laboratory. Repeatability and intermediate precision for the related impurities in SAMe were found to be < 6.0 % R.S.D confirming good precision of the method (Table 2)

Limit of detection (LOD) and limit of quantification (LOQ):
The LOD and LOQ for Impurities A - F were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentration. Precision study was also carried at the LOQ level by injecting six replicate injections of solution containing Impurities A - F, and calculated the percentage R.S.D of the area (Table 2)

Table 2. Validation data of the developed method.

Parameter	Imp A	Imp B	Imp C	Imp D	Imp E	Imp F
DL (%)	0.006	0.004	0.006	0.009	0.010	0.017
QL (%)	0.020	0.013	0.020	0.030	0.035	0.056
Precision (% RSD)#	0.01	0.01	1.23	0.01	0.01	0.01
Intermediate precision (% RSD)#	0.01	0.01	1.63	0.01	0.01	5.50
Accuracy [a] (% recovery) at						
QL	101.53	103.89	116.67	90.44	102.32	108.69
50%	97.08	112.01	109.73	98.09	98.39	103.74
100%	98.48	103.78	98.32	97.96	99.15	100.96
150%	97.65	109.28	98.36	100.51	100.86	102.15

[a] *Carried at QL, 50%,100% and 150% level with respect to specification 1.5 % for A,C,D,E,F and 3.0% for B.*

Robustness:
In order to demonstrate the robustness of the method, system suitability parameters were verified by making deliberate changes in the chromatographic conditions, viz. change in flow rate by ± 0.2 mL/min, change in the organic composition of mobile phase (± 2% absolute) and column oven temperature at 35°C.The method was demonstrated to be robust over an acceptable working range of its HPLC operational parameters.

CONCLUSION

The gradient RP-LC method developed for related substance determination SAMe is precise, accurate and specific. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method is stability indicating and can be used for the routine analysis of production samples and also to check the stability of bulk samples of SAMe.

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