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NMR Regulatory analysis: determination and characterization of S-adenosyl-L-methionine in dietary supplements

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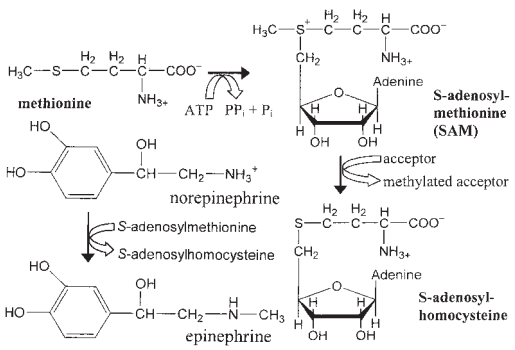
^{1}H NMR methodology is described for the determination and characterization of the dietary supplement *S*-adenosyl-L-lmethionine (SAM), recently introduced to the US market, utilizing a 400 MHz spectrometer without the need of pure reference standards. The developed methodology is able to assess chemical structure, differentiate between biologically-active (*S*)-diastereomer and biologically-inactive (*R*)-diastereomer, and determine the ratio of each in the dietry supplement formulation. The determination of the percentage of declared SAM was based on the integrals for the methyl proton of 2-methyl-2-propanol served as an internal standard at δ 1.24 and the methine proton $H_{1'}$ of SAM ribose ring at δ 6.06. The percentage of the active diastereomer was calculated from the relative intensities of the sulfonium methyl singlets corresponding to the major component (*S*)-diastereomer at δ 2.98 and the minor (*R*)-counterpart at δ 2.93. The accuracy was established by analyzing synthetic mixtures of the analyte and the internal standard. Excellent agreement was verified between the assay results and the quantities of analyte in the mixture. The mean ± SD recovery values for SAM and its (*R*)-diastereomer impurity from a set of 10 synthetic mixtures were 99.6 ± 0.8% and 22.5 ± 0.1%, respectively. Using 10 lots, the percentage of SAM ranged from 0 to 110.7% of the declared value and the percentage of the active (*S*)-diastereomer ranged from 0 to 82.3%.

1. Introduction

S-Adenosyl-L-methionine (SAM), the only natural sulfonium compound present in mammals, is normally manufactured by combining the essential amino acid methionine with adenosyltriphosphate (ATP). It is an important biological agent in the human body, participating in over 40 essential biochemical reactions of cellular functions (Salvatore et al. 1977). It is involved in enzymatic transmethylations of lipids, proteins, RNA and DNA, in which a methyl group is transferred from SAM to a wide variety of methyl acceptors, the biosynthesis of aliphatic polyamine and the trans-sulfuration reactions (Paik et al. 1975; Giulidori et al. 1984; Prased et al. 1985). Immunological effects (Chiang 1981) and cellular differentiation (Crooks et al. 1979; Zimmerman et al. 1984) can be triggered by perturbation of SAM metabolic pathways. Conversion of methionine to SAM and an example of methylation process are shown in the Scheme.

Scheme



The well-established biomedical roles and pharmacological effects of this sulfonium compound have rationalized a great interest in its therapeutic use (Friedle et al. 1989). It has been claimed that it prevents the reduction of phospholipids methylation, maintains membrane fluidity, protects the liver against elusive agents and helps in depressive syndromes and in osteoarthritis (Cimino et al. 1984; Osman et al. 1993; Varela-Moreiras et al. 1995). It has been reported that it participates in detoxification reactions and in the synthesis of brain chemicals, antioxidants, joint tissue structures, and many other important components (Bottiglier et al. 1994; Chiang et al. 1996). It has been documented that it plays an important role in the biochemistry connected with schizophrenia and depression (Fazio et al. 1974; Bottiglier et al. 1982). It raises levels of dopamine, an important neurotransmitter in mood regulation (Fava et al. 1990), and higher levels in the brain are associated with drug treatment of depression (Bell et al. 1994). In fact, it was found to be effective for depression

in some (Kagan et al. 1990; Salmaggi et al. 1993; Bell et al. 1994; Bressa 1994), though not all (De Vanna and Rigamonti 1992; Fava et al. 1992; Fava et al. 1995), double-blind studies. Also, it was reported that it possesses anti-inflammatory and analgesic properties (Marcolongo et al. 1985; Glorioso et al. 1985; Montrone et al. 1985; Schumacher 1987; Harmand et al. 1987; Muller-Fassbender 1987; Vetter 1987; Maccagno 1987; Caruso and Pietrogrande 1987; Domljan et al. 1989; Konig et al. 1995). SAM has shown a rapid increase in its use as a dietary supplement since being introduced into the US market in 1999. In the US, it is marketed for improved mobility and mood enhancement. However, it has a longer history in other countries as a drug for the treatment of various liver diseases, pain associated with arthritis, and depression. SAM is available commercially as stable sulfate and *p*-toluenesulfonate salts (Fiecchi 1976; Barabato et al. 1989). The absolute configuration of the biologically active form was designated as (*S*)-*S*- (Conforth et al. 1977), where the designations refer to the alpha-carbon and the sulfur, respectively.

Serious consequences for consumers are anticipated because of lack of quality assurance on dietary supplements. Adulterations with prescription medicines, misidentifications and substitutions and false labeling are considered some of the problems in SAM as dietary supplements. Methods for quality assurance must be able to stereospecifically determine the active ingredient, its counter ions and any possible related products. Various liquid chromatographic methods have been used for the determination of SAM in body fluids, tissues and solid form extracts. Some are based on using ion-pair liquid chromatography combined with ultraviolet detection (Wagner et al. 1986; Cools et al. 1990; Perna et al. 1993; Balaghi et al. 1993; Perna et al. 1995; She et al. 1995) and others based on the conversion of SAM to the fluorescent derivatives before or after column separation followed by fluorescence detection (Weir et al. 1992; Loehrer et al. 1996a and 1996b; Loehrer et al. 1997; Loehrer et al. 1998). Ion-pair reagent in mobile phase showed decrease in reproducibility of the methods. Fluorescence methods provided adequate sensitivity but have the disadvantage of being very laborious and time-consuming. SAM was also measured in trichloroacetate using electrochemical detection (Melnyk et al. 2000) and by stable-isotope dilution and tandem mass spectrometry (Struys et al. 2000). Nuclear magnetic resonance spectroscopy for the quantitative analysis of SAM in tablets with sodium trimethylsilylpropionate (TSP) as internal standard has been described (Revelle et al. 1995). The method was based on the measurement of the signals of the sulfonium methyl protons of SAM and that of the internal standard, sodium trimethylsilylpropionate-d_4. The method suffered from at least three drawbacks. First, integration of the relevant resonance signal is not very accurate because of the close proximity of this signal to that of γ protons of the methionine side chain of SAM which appeared as two sets of 6-line multiplets. Second, TSP was a poor choice as internal standard because it required 30 hours drying and applying a factor to correct for water content. Third, it is well known that losses of TSP from solution due to adsorption onto the walls of the NMR tube (Larive et al. 1997) disqualify its use as internal standard for quantitative analysis. In addition, the method made no provisions for structural assignments of SAM. The method described here could be used to achieve speed, accuracy and simplicity for specific characterization and quantification of SAM in commercial dietary supplement formulations.

2. Investigations, results and discussion

All the proton resonances of SAM were assigned based on chemical shift values, spin multiplicities and decoupling experiments. The assignments were facilitated by comparing ^{1}H NMR spectra of adenine, adenosine, and L-methionine in D_2O with that of SAM in D_2O, (Figs. 1–4). The α proton of the methionine side chain of SAM appeared as a triplet at δ 3.78 with $J_{\alpha\beta} = 7.39$ Hz, the β protons come out as a quartet centered at δ 2.33, and the γ protons emerged as two sets of 6-line multiplets centered at δ 3.45 and δ 3.67. Homonuclear decoupling of the protons turned the β protons signal to a triplet with $J_{\beta\gamma} = 7.39$ Hz, and the γ protons resonance unchanged. Irradiation of the β protons gave a singlet for the resonance and an AB quartet with $J_{\gamma\gamma} = -11.19$ Hz. This is because the β protons are equivalent but the γ protons,

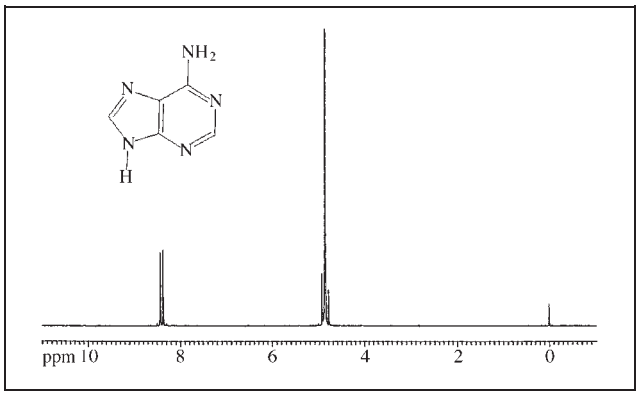


Fig. 1: 400 MHz ^{1}H NMR spectrum of adenine hydrochloride in D_2O

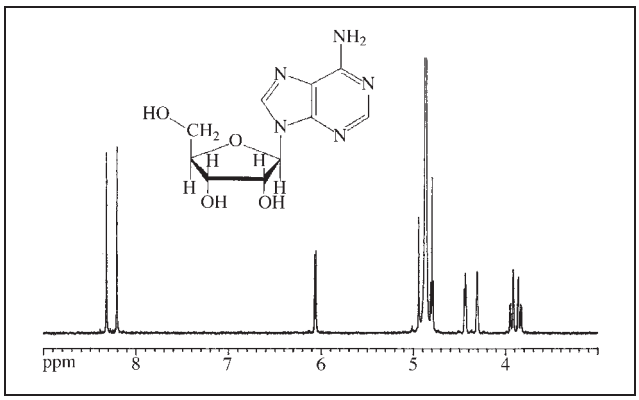


Fig. 2: 400 MHz ^{1}H NMR spectrum of adenosine hydrochloride in D_2O

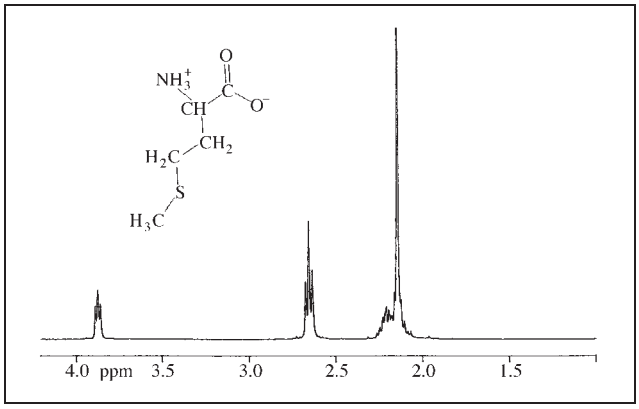


Fig. 3: 400 MHz ^{1}H NMR spectrum of L-methionine in D_2O

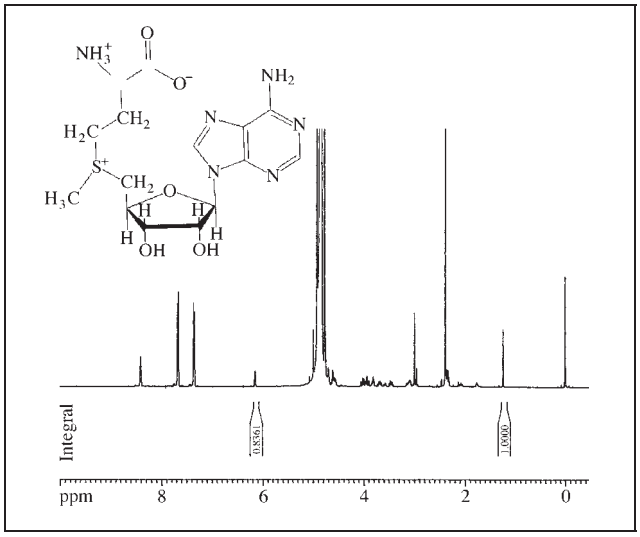


Fig. 4: 400 MHz ^{1}H NMR spectra of SAM and TBA in D_2O

next to the asymmetric sulfonium group, are nonequivalent. Sulfonium methyl group protons emerged as a sharp singlet at δ 2.98. It is accompanied by a second smaller upfield singlet at δ 2.93, regardless of the commercial source, was attributed to the *S*-methyl proton of the diastereomer (*R*)-*S*-adenosyl-L-methionine. It cannot be due to any known degradation product because this would exhibit additional resonances which were not observed. The samples of SAM used were stable at room temperature, and the relative intensities of these two peaks for freshly prepared samples did not change over the course of several days. The ribose ring protons of SAM constituted a six-spin ABKMNX system where $A = H_{5''}$, $B = H_{5'}$, $K = H_{4'}$, $M = H_{3'}$, $N = H_{2'}$, and $X = H_{1'}$. $H_{1'}$ appeared as a doublet centered at δ 6.06 with $J_{1'2'} = 6.2$ Hz. $H_{2'}$, $H_{3'}$, and $H_{4'}$ signals were centered at δ 4.8, δ 4.30, and δ 4.43, respectively, with coupling constants as follows: $J_{2'3'} = 5.32$, $J_{3'4'} = 0.05$, $J_{4'5'} = 9.5$, and $J_{4'5'} = 2.4$ Hz. The $H_{5'}$, $H_{5''}$ and $H_{4'}$ protons which comprised a three-spin ABX system, appeared at δ 3.84, δ 3.92 and δ 4.30 with $J_{4'5'} = 9.5$, $J_{4'5''} = 2.4$, and $J_{5'5''} = 12.8$ Hz. The adenine ring protons of SAM, H_2 and H_8, appeared as 2 singlets at δ 8.20 and δ 8.31, respectively. The assignments have been confirmed by computer simulation. Adenine, adenosine, L-methionine and SAM in D_2O ^{1}H NMR spectral data are presented in Tables 1–4, respectively.

Table 1: ^{1}H NMR spectral assignments of adenine hydrochloride in D_2O

Chemical shift[a], δ	Multiplicity	Number of protons	Assignment
8.37	*s*	1	H_2
8.42	*s*	1	H_8

[a] Chemical shifts were measured vs. internal TSP, sodium 3-(trimethylsilyl) tetradeuterio propionate

Table 2: ^{1}H NMR spectral assignments of adenosine in D_2O

Chemical shift[a], δ	Multiplicity	Coupling constant, (Hz)	Number of protons	Assignment
3.92	*m*	$J_{4'5''} = 2.4$	1	$H_{5''}$
3.84	*m*	$J_{5'5''} = -12.8$	1	$H_{5'}$
4.43	*m*	$J_{4'5'} = 9.5$	1	$H_{4'}$
4.30	*m*	$J_{3'4'} = <0.5$	1	$H_{3'}$
4.80	*m*	$J_{2'3'} = 5.32$	1	$H_{2'}$
6.06	*d*	$J_{1'2'} = 6.2$	1	$H_{1'}$
8.20	*s*		1	$H_{2'}$
8.31	*s*		1	$H_{8'}$

[a] Chemical shifts were measured vs. internal TSP, sodium 3-(trimethylsilyl) tetradeuterio propionate

Table 3: ^{1}H NMR spectral assignments of L-methionine in D_2O

Chemical shift[a], δ	Multiplicity	Coupling constants, (Hz)	Number of protons	Assignment
3.78	*t*	$J_{\alpha\beta} = 5.3$	1	H_α
2.14	*m*	$J_{\beta\gamma} = 7.6$ $J_{\alpha\beta'} = 6.9$	2	$H_{\beta\beta'}$
2.65	*t*	$J_\gamma = -11.19$	2	H_γ
2.14	*s*		3	H_{SCH3}

[a] Chemical shifts were measured vs. internal TSP, sodium 3-(trimethylsilyl) tetradeuterio propionate

The percentage of declared SAM is calculated by measuring the intensities of the signals for the $H_{1'}$ of SAM (d, δ 6.06) and the methyl protons of the internal standard t-butyl alcohol (s, δ 1.24), Fig. 4. The functional portion of the SAM molecule is the sulfonium methyl group, and the extensively held view is that the naturally occuring and the active SAM is only the (*S*)-diastereomer, then the relative intensity of the two signals attributed to the protons of this group corresponding to the major component (*S*)-*S*-adenosyl-L-methionine (s, δ 2.98) and the minor diastereomer (*R*)-(*S*)-adenosyl-L-methionine (s, δ 2.93) is a direct

Table 4: ^{1}H NMR spectral assignments of SAM in D_2O

Chemical shift[a] δ	Multiplicity	Coupling constants (Hz)	Number of protons	Assignment
3.78	*t*	$J_{\alpha\beta} = 6.59$	1	H_α
2.33	*tt*	$J_{\beta\gamma} = 7.39$	2	H_β
3.67	*m*	$J_{\gamma\gamma'} = -11.19$	1	$H_{\gamma'}$
3.45	*m*		1	H_γ
2.98	*s*		3	(−)-diastereomer H_{SCH3}
2.93	*s*		3	(+)-diastereomer H_{SCH3}
3.92	*m*	$J_{4'5''} = 2.84$	1	$H_{5''}$
3.84	*m*	$J_{5'5''} = -12.8$	1	$H_{5'}$
4.43	*m*	$J_{4'5'} = 9.5$	1	$H_{4'}$
4.30	*m*	$J_{3'4'} = <0.5$	1	$H_{3'}$
4.80	*m*	$J_{2'3'} = 5.32$	1	$H_{2'}$
6.11	*d*	$J_{1'2'} = 4.36$	1	$H_{1'}$
8.20	*s*		1	H_2
8.31	*s*		1	H_8

[a] Chemical shifts were measured vs. internal TSP, sodium 3-(trimethylsilyl) tetradeuterio propionate

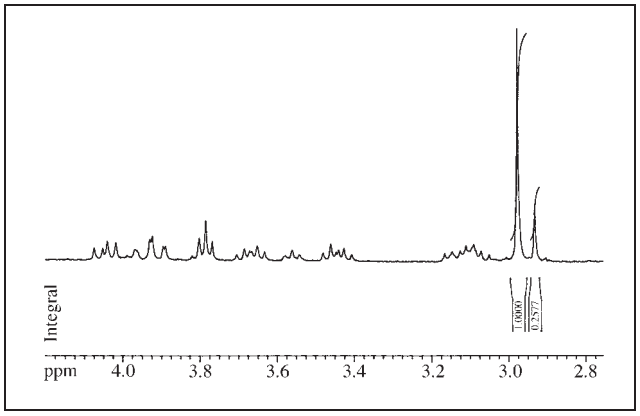


Fig. 5: 400 MHz ^{1}H NMR spectra of 80% of (S)-SAM and 20% (R)-counterpart

measure of activity. Fig. 5 shows the ^{1}H NMR spectra of 80% active (*S*)-diatereomer and 20% (*R*)-counterpart.

As a test for accuracy, the proposed method was used to analyze a set of ten mixtures made from known quantities of SAM and the internal standard. The mean ± SD recovery values for SAM and its (*R*)-diastereomer impurity were 99.6 ± 0.8% and 22.5 ± 0.1%, respectively. From the results summarized in Table 5, it was concluded that

Table 5: Results of the assay of SAM and (R)-diastereomer in synthetic mixtures by ^{1}H NMR spectroscopy [a]

Mix. No.	Internal standard added [b] (mg)	SAM			(*R*)-Diasteromer found (%)
		(mg) added	(mg) found	(mg) recovery (%)	
1	0.66	3.57	3.52	98.6	22.4
2	0.66	9.02	8.94	99.1	22.4
3	0.66	7.04	7.06	100.3	22.6
4	0.66	4.56	4.51	98.9	22.6
5	0.66	8.51	8.42	98.9	22.5
6	0.66	6.07	6.02	99.2	22.4
7	0.66	7.51	7.56	100.6	22.6
8	0.66	6.47	6.46	99.2	22.6
9	0.66	5.54	5.55	100.2	22.4
10	0.66	8.03	8.09	100.7	22.5
Mean				99.6	22.5
SD				0.8	0.1

[a] Recoveries were calculated from (100 × amount found)/amount added.
[b] Added from a 89.38 μM solution of TBA in D_2O (25 μL)

Table 6: Results of the assay of SAM and its active (*S*)-diastereomer in 200 mg caplets from various lots by ^{1}H NMR spectroscopy

Sample No.	SAM		(*S*)-Diastereomer, activity (%)
	found (mg)	declared (%)	
1	181.0	90.5	77.5
2	177.6	88.8	79.3
3 [a]	0		
4	196.4	98.2	82.3
5	210.6	105.3	63.2
6	199.2	99.7	79.5
7	221.4	110.7	66.7
8	191.8	95.9	78.4
9	215.6	107.8	60.1
10 [a]	0		
Mean	159.4	79.7	58.7
Range	0–221.4	0–110.7	0–82.3

[a] Sample was found to contain caffeine and methionine instead of SAM as labeled

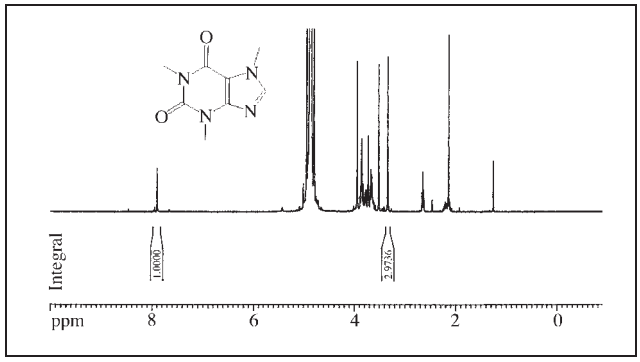


Fig. 6: 400 MHz ^{1}H NMR spectra of TBA and commercial 200 mg caplets in D_2O contained caffeine and methionine instead of the claimed SAM

the accuracy of the method was maintained at the various levels of the analytes present and the fixed amount of internal standard added. Ten lots of commercial caplets of SAM were analyzed by ^{1}H NMR spectroscopic method for their contents of active and inactive diastereomers. Table 6 shows that the percentage of SAM ranged from 0 to 110.7% of the declared value and the percentage of the active (*S*)-diastereomer ranged from 0 to 82.3%. It is impossible to say whether the (*R*)-diastereomer in commercial samples is the result of some reaction during purification or whether both diastereomers are biosynthesized under the conditions used by the supplier. It cannot be due to any known degradation product because these would exhibit additional resonances which were not observed. There was no change noticed in the ratio of the active (*S*)-diastereomer to the inactive (*R*)-diastereomer at room temperature for several hours. The stability and lack of spontaneous degradation of SAM *p*-toluenesulfonate salt can be attributed to the presence of large anion size (Fiecchi 1976). The large anion increases steric hindrance and the intra-molecular attack responsible for spontaneous degradation cannot occur (Barbato et al. 1989).

In conclusion, the ^{1}H NMR spectroscopic method described in this report will permit the identification and assay of the active diastereomer of SAM in the presence of its inactive counterpart. Problems commonly encountered in dietary supplements such as adulterations with prescription medicines, misidentifications and substitutions and false labeling can also be addressed. Fig. 6 shows the ^{1}H NMR spectrum of commercial 200 mg caplets of SAM which were found to contain caffeine and methionine instead of the claimed active ingredient. Another advantage is that the method does not rely on the use of pure reference standards.

3. Experimental

3.1. Apparatus

All ^{1}H NMR spectra were obtained on a model AMX-400 spectrometer operating at 400.13 MHz (Bruker Instruments, Inc., Billerica, MA) and 28 °C. The chemical shifts were referenced to TSP, sodium 3-(trimethylsilyl) tetradeuteriopropionate.

3.2. Materials

Deuterium oxide, 99.8 atom% D, sodium 3-(trimethylsilyl) tetradeuteriopropionate (TSP) and 2-methyl-2-propanol, 99.5 + %, deuterium chloride, 37 wt% in D_2O and 99.5 atom%, and D,L-methioine, 99 + %, were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). *S*-Adenosyl-L-methionine *p*-toluenesulfonate, 92%, was obtained from Sigma Chemical Co. (St.Louis, MO). Adenosine free base and adenine hydrochloride were purchased from ICN Biomedicals, Inc. (Irvine, CA, USA).

3.3. Samples

S-Adenosyl-L-methionine *p*-toluenesulfonate caplets (200 mg/caplet) were obtained from local commercial sources.

3.4. Standard solutions

Stock solutions of 2-methyl-2-propanol (TBA) (6.63 mg/mL) were prepared in D_2O, transferred to glass vials, and immediately crimper-sealed with Teflon-coated rubber septa and aluminum seals. When needed, these solutions were withdrawn through the septa by means of a liquid-tight micro-liter syringe.

3.5 Assay procedure

Synthetic mixtures containing *S*-adenosyl-L-methionine *p*-toluenesulfonate and TBA, were prepared by first adding an accurately weighed quantity of *S*-adenosyl-L-methionine *p*-toluenesulfonate to a glass vial, followed by 0.100 mL TBA stock solution, prepared in D_2O. Volume was adjusted to 4.0 mL and the vial immediately crimped-sealed with Teflon-coated rubber septa and aluminum seal. The solution was mixed by means of vortex shaker for about 1 min. Approximately, 0.75 mL was transferred to a 5 mm NMR tube, which was placed in spectrometer, and the spectrum obtained.

To analyze commercial *S*-adenosyl-L-methionine *p*-toluenesulfonate caplets, a group of 20 caplets was accurately weighed, placed in a glass mortar, and ground to a fine powder. An accurately weighed portion of the powder, equivalent to about 32 mg of *S*-adenosyl-L-methionine (51 mg of *S*-adenosyl-L-methionine *p*-toluenesulfonate), was transferred to a to a glass vial, added 0.100 mL TBA stock solution, dissolved in D_2O. Volume was adjusted to 4.0 mL and the vial immediately crimped-sealed with Teflon-coated rubber septa and aluminum seal. The solution was mixed by means of vortex for 1 min, and next centrifuged at 3000 rpm for 5 min. Approximately, 0.75 mL from the clear supernatant was transferred to a 5 mm NMR tube, and then placed in the spectrometer, and the spectrum obtained.

The quantity of *S*-adenosyl-L-methionine *p*-toluene sulfonate is calculted by measuring the intensity of the signals for the H1′ of the drug (d, δ 6.06) and the methyl protons of the internal standard t-butyl alcohol (s, δ 1.24) and using the following equation:

$$\begin{gathered}S\text{-Adenosyl-L-methionine } p\text{-tolune sulfonate,}\\ \text{mg} = [\text{As}/\text{At}] \times [\text{EWt}/\text{EWs}] \times \text{Mt}\end{gathered}$$

where As is the integral value of *S*-adenosyl-L-methionine *p*-toluene sulfonate, At is the integral value of the internal standard, EWs is the formula weight of *S*-adenosyl-L-methionine *p*-toluene sulfonate divided by the number of protons (i.e., 588/1 = 588), EWt is the formula weight of the internal standard divided by the number of absorbing protons (i.e., 74.12/9 = 8.24), and Mt is the amount of internal standard added, mg.

The percentage of the active diastereomer was calculated from the relative intensities of the sulfonium methyl singlets corresponding to the major component (*S*)-diastereomer at δ 2.98 and the minor (*R*)-counterpart at δ 2.93.

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