



SHORT COMMUNICATION

Determination of genotoxic alkyl methane sulfonates and alkyl paratoluene sulfonates in lamivudine using hyphenated techniques

N.V.V.S.S. Raman^a, A.V.S.S. Prasad^a, K. Ratnakar Reddy^a, K. Ramakrishna^{b,*}

^aHetero Drugs Ltd. (R&D), Plot No. B, 80 & 81, APIE, Balanagar, Hyderabad-500 018, India

^bGITAM Institute of Science, GITAM University, Visakhapatnam-530 045, India

Received 19 November 2011; accepted 6 March 2012

Available online 14 March 2012

KEYWORDS

Genotoxins;
Alkyl methane sulfonates;
Alkyl paratoluene sulfonates;
Lamivudine;
Hyphenated techniques

Abstract Two highly sensitive methods for the determination of genotoxic alkyl methane sulfonates (AMSs) and alkyl paratoluene sulfonates (APTSS) in lamivudine using hyphenated techniques have been presented. AMSs were determined by GC–MS method using GSBP-INOWAX (30 m × 0.25 mm × 0.25 μm) column. Temperature program was set by maintaining at 100 °C initially for 3 min, then risen to 220 °C at the rate of 15 °C/min and maintained at 220 °C for 16 min. N,N-dimethyl formamide was used as diluent. APTSSs were determined by LC-MS using Zorbax, Rx C8, 250 mm × 4.6 mm, 5 μm column as stationary phase. 0.01 M ammonium acetate is used as buffer. The mixture of buffer and methanol in 75:25 (v/v) ratio was used as mobile phase A and mixture of buffer and methanol in 5:95 (v/v) ratio was used as mobile phase B. The gradient program (T/%B) was set as 0/28, 16/50, 17/100, 23/100, 27/28 and 40/28. Both the methods were validated as per International Conference on Harmonization guidelines. Limit of quantitation was found 1.5 μg/mL for AMSs and was in the range of 1.0–1.5 μg/mL for APTSSs.

© 2012 Xi'an Jiaotong University. Production and hosting by Elsevier B.V.

Open access under [CC BY-NC-ND license](#).

1. Introduction

Synthesis of drug substances often involves the use of reactive reagents and hence, these reagents may be present in the final drug substances as impurities. Such chemically reactive impurities may have unwanted toxicities, including genotoxicity and carcinogenicity and are to be controlled based on the maximum daily dose [1]. These limits generally fall at low μg/mL levels and hence conventional HPLC, GC methods (or final drug substance methods) are not suitable for their determination. Hyphenated techniques like GC–MS and LC–MS combine physical separation capabilities of chromatography (GC or HPLC) with the mass analysis capabilities of mass

*Corresponding author. Tel.: +91 98662 34551; fax: +91 0891 2790032.
E-mail address: karipeddi_rk@yahoo.com (K. Ramakrishna).



spectrometry and have high sensitivity and specificity over conventional HPLC and GC methods. Their applications are oriented towards the potential identification and quantitation of trace level of impurities in drug substances [2].

Lamivudine (LMD) is chemically known as (2R-Cis)-4-Amino-1-[2-(hydroxy methyl)-1,3-oxathiolan-5-yl]-2(1H)-pyrimidinone. It is used to treat HIV (Type 1) and hepatitis B [3]. In the manufacturing process of LMD, methane sulfonic acid (MSA) and paratoluene sulfonic acid (PTSA) are used as reagents and three alcohols (viz. methanol, ethanol and isopropanol) are used as solvents and hence genotoxic methyl methane sulfonate (MMS), ethyl methane sulfonate (EMS) and isopropyl methane sulfonate (IPMS), methyl paratoluene sulfonate (MPTS), ethyl paratoluene sulfonate (EPTS) and isopropyl paratoluene sulfonate (IPPTS) may exist as impurities in lamivudine drug substance. Based on maximum daily dose of LMD (300 mg/day), these are to be controlled at a limit of 5 µg/mL.

In literature, some analytical methods using hyphenated techniques for the determination of AMSs [4–7], APTSs [5,6,8] and LMD [9–13] were reported. However, no method was reported for the determination of AMSs and APTSs in LMD. Hence, the present work is aimed towards the development of rapid, specific and robust methods for the determination of AMSs and APTSs in LMD at trace level concentration.

2. Experimental

2.1. Chemicals and reagents

MMS, EMS and IPMS were purchased from Acros organics, Geel, Belgium. MPTS and EPTS were purchased from Aarti Drugs Ltd., Mumbai, India. N,N-dimethyl formamide, ammonium acetate, acetonitrile and methanol were procured from Merck, India. IPPTS and pure samples of LMD were obtained from synthetic division of Hetero Drugs Ltd. (R&D), Hyderabad, India.

2.2. Preparation of stock solutions

N,N-dimethyl formamide was used as diluent in GC–MS method. MMS, EMS and IPMS stock solutions were prepared by dissolving 10 mg each individually in 10 mL of diluent. The mixture solution, 1000 µg/mL with respect to 200 mg/mL of LMD, was prepared by diluting the appropriate volumes of above stock solutions with diluent.

The mixture of water and acetonitrile in the ratio of 65:35 v/v was used as diluent in LC-MS method. MPTS, EPTS and IPPTS stock solutions were prepared by dissolving 10 mg each individually in 10 mL of diluent. The mixture solution, 1000 µg/mL with respect to 50 mg/mL of LMD, was prepared by diluting the appropriate volumes of above stock solutions with diluent as above. A blend solution was also prepared by spiking 1000 µg/mL of APTSs to 50 mg/mL of LMD and is used for method development.

2.3. GC–MS conditions

GC–MS analysis was carried out on GCMS-QP2010 system (Shimadzu Corporation, Japan) having GCMS solution software. The instrument was run in EI mode. GSBP-INOWAX

column (30 m × 0.2 mm i.d. × 0.25 µm film, Agilent Technologies, USA) was used as stationary phase. 1.5 µL volume with 1:5 split inlet was selected for injection. The GC oven temperature program was set by maintaining at 100 °C initially for 3 min, then risen to 220 °C at the rate of 15 °C/min maintained at 220 °C for 16 min. The injection temperature, GC–MS interface temperature and ion source temperature were 200, 240 and 240 °C, respectively. Helium was used as the carrier gas with a flow rate of 1.46 mL/min. The ionizing energy was 70 eV. The mass detector gain is 1.5 kV.

2.4. LC–MS conditions

LC–MS analysis was carried out on Shimadzu LCMS-2010 EV system (Shimadzu Corporation, Japan) having LCMS solution software in electro spray ionization (positive) mode. Zorbax, Rx C8 column (250 mm × 4.6 mm, 5 µm, Agilent Technologies, USA) was used as stationary phase. 0.01 M ammonium acetate is used as buffer. The mixture of buffer and methanol in 75:25 (v/v) ratio was used as mobile phase A and that of buffer and methanol in 5:95 (v/v) ratio was used as mobile phase B. The gradient program (T/%B) was set as 0/28, 16/50, 17/100, 23/100, 27/28 and 40/28. The flow rate of the mobile phase was kept at 1.0 mL/min. The injection volume was set as 50 µL. Column oven temperature and auto sampler temperature were set as 50 °C and 20 °C, respectively. Interface, curve dissolution line (CDL) and detector voltages are 4.5 kV, 5.0 V and 1.75 kV, respectively. Interface, CDL and heat block temperatures were 250, 250 and 200 °C, respectively. Nubulizing gas flow was 1.5 L/min.

3. Results and discussion

3.1. GC–MS method development

LMD is soluble in N,N-dimethyl formamide (DMF) and hence it was used as diluent. AMSs mixture solution was initially run through using DB-1 column (100%-Dimethylpolysiloxane). The resolution between MMS and IPMS is not adequate in this column. Then, this column was replaced by DB-5 column and the same result was found. Finally, GSBP-INOWAX column was used and good resolutions were observed. An optimum injection volume of 1.5 µL was chosen. The split ratio was fixed as 1:5 depending on the detector response. An initial column temperature of 100 °C was found to be optimum. The elution order was observed from the total ion chromatogram (Fig. 1) in SCAN mode using AMSs mixture (1.5 µg/mL each) and the individuals were also confirmed using the National Institute of Standard Technology mass spectral library. Validation was done in Selective Ion Monitoring (SIM) monitoring for m/z ions at 110 for MMS, 124 for EMS and 138 for IPMS.

3.2. LC–MS method development

A blend solution containing APTSs and LMD was run in literature method [8]. LMD eluted too early and hence the flow rate of the mobile phase was reduced from 1.5 mL/min to 1.0 mL/min. In this condition LMD eluted at an optimum retention time, but the retention times of APTSs were drastically increased. Hence, the gradient program (T/%B) was fine tuned to 0/28,

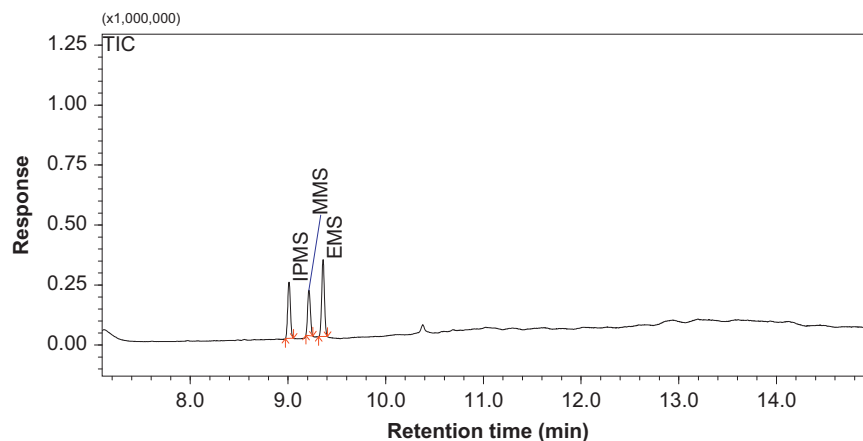


Figure 1 GC-MS total ion chromatogram of AMSs.

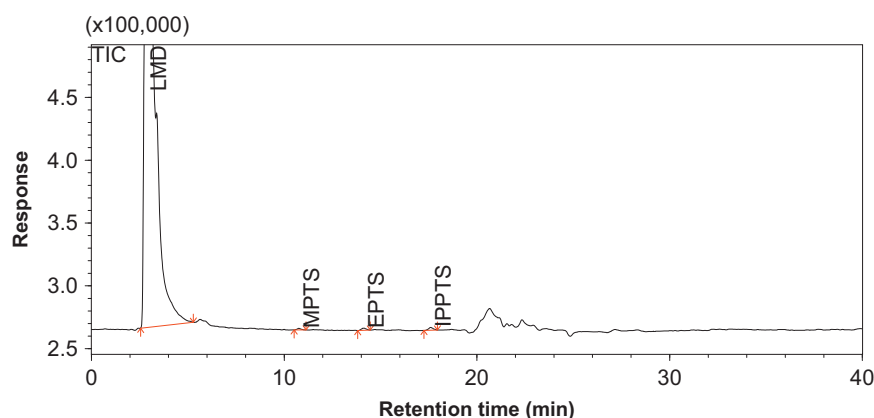


Figure 2 LC-MS total ion chromatogram of APTs.

16/50, 17/100, 23/100, 27/28 and 40/28 and optimum retention times were achieved for APTs. The elution order was observed from the total ion chromatogram (Fig. 2) in SCAN mode using lamivudine solution spiked with MPTS (1.5 µg/mL), EPTS (1.5 µg/mL) and IPPTS (1.0 µg/mL). Validation was done in Selective Ion Monitoring (SIM) monitoring for $[M+NH_4]^+$ ions at 204 for MPTS, 218 for EPTS and 232 for IPPTS.

3.3. Method validation

The developed methods were validated as per ICH guidelines [14] in terms of specificity, limit of detection (LOD), limit of quantitation (LOQ), precision, linearity, accuracy, robustness and system suitability and the data are presented in Table 1 (GC-MS) and Table 2 (LC-MS).

The specificity of the developed GC-MS and LC-MS methods was indicated by showing the m/z peaks in GC-MS method as 110 for MMS, 124 for EMS and 137 for IPMS and $[M+NH_4]^+$ peaks as 204 for MPTS, 218 for EPTS and 232 for IPPTS.

In GC-MS method, AMSs solutions (1000 µg/mL each) with respect to 200 mg/mL of LMD and in LC-MS method, APTs solutions (1000 µg/mL each) with respect to 50 mg/mL of LMD were injected separately and S/N ratios were recorded. These solutions were further diluted to achieve the signal-to-noise (S/N)

ratios at about 3 and 10 for determining LOD and LOQ, respectively for both the methods.

The precision of the methods was checked by injecting LOQ solutions for six times. The values of RSDs for areas of each AMSs (in GC-MS) and APTs (in LC-MS) were calculated. The intermediate precision of the methods was also verified on six different days in the same laboratory using the LOQ level solutions. The low RSD values ensured the precision of the developed methods.

Linearity test solutions for AMSs and APTs were prepared individually at six concentration levels in the range of LOQ to 120% of the specification level viz. 5 µg/mL. LOQ and sixth levels were injected six times and other four levels were injected thrice. The average peak areas versus concentrations were subjected to least-squares linear regression analysis. The derived correlation coefficients were above 0.995 indicating the best fitness of the linearity curves of the developed methods.

Standard addition experiments were conducted in triplicate preparations to determine accuracy of the methods at LOQ level and recoveries of all the genotoxins were determined. The recoveries were found to be in the accepted range.

The robustness of GC-MS was illustrated by getting the identical retention times and peak areas of AMSs in the varied GC conditions of $\pm 5\%$ on the carrier gas flow, $\pm 5^\circ\text{C}$ on the initial oven temperature, $\pm 1^\circ\text{C}/\text{min}$ on the ramp rate. Similarly, the robustness of LC-MS method was ensured by getting the resolution between any two APTs to be greater than 2.0, when

Table 1 Validation data of GC–MS method for the determination of MMS, EMS and IPMS.

Parameter	MMS	EMS	IPMS
LOD ($\mu\text{g/mL}$)	0.5	0.5	0.5
LOQ ($\mu\text{g/mL}$)	1.5	1.5	1.5
Precision at LOQ level (RSD, %)	1.99	1.36	1.55
Precision at sixth level (RSD, %)	0.84	0.90	0.87
Intermediate precision at LOQ (RSD, %)	2.08	1.58	1.86
Linearity range ($\mu\text{g/mL}$)	1.5–6.0	1.5–6.0	1.5–6.0
Correlation coefficient	0.999	0.999	0.999
Slope	60332	97982	73058
Intercept	1524	28001	13134
Accuracy at LOQ (recovery, %)			
Preparation-1	102.0	102.4	95.0
Preparation-2	104.0	104.8	95.0
Preparation-3	105.3	106.4	107.1

Abbreviations:

MMS=Methyl methane sulfonate,

EMS=Ethyl methane sulfonate,

IPMS=Isopropyl methane sulfonate.

Table 2 Validation data of LC–MS method for the determination of MPTS, EPTS and IPPTS.

Parameter	MPTS	EPTS	IPPTS
LOD ($\mu\text{g/mL}$)	0.6	0.6	0.3
LOQ ($\mu\text{g/mL}$)	1.5	1.5	1.0
Precision at LOQ level (RSD, %)	1.60	1.62	1.20
Precision at sixth level (RSD, %)	0.70	0.71	1.80
Intermediate precision at LOQ (RSD, %)	1.14	1.89	1.35
Linearity range ($\mu\text{g/mL}$)	1.5–6.0	1.5–6.0	1.0–6.0
Correlation coefficient	0.999	0.999	0.999
Slope	23310	23064	38099
Intercept	569	824	766
Accuracy at LOQ (recovery, %)			
Preparation-1	98.1	101.6	101.0
Preparation-2	97.9	98.3	98.8
Preparation-3	94.5	103.3	102.7

Abbreviations:

MPTS=Methyl paratoluene sulfonate,

EPTS=Ethyl paratoluene sulfonate,

IPPTS=Isopropyl paratoluene sulfonate.

mobile phase flow rate (± 0.2 mL/min), organic solvent ratio in both mobile phases A and B ($\pm 2\%$) and column temperature (± 5 °C) were deliberately varied.

The solution stability of APTSs in diluent in LC–MS method was determined by leaving APTSs mixture solution at specification level in a tightly capped volumetric flask at room temperature for 48 h and measuring the amounts of the APTSs for every 6 h. All the APTSs were found to be stable up to 48 h.

The system suitability of both the methods was ensured by getting the %RSD less than 10.0 for six injections of all the AMSs in GC–MS method and APTSs in LC–MS method at specification level.

4. Conclusions

GC–MS and LC–MS methods that can quantify genotoxic alkyl methane sulfonates and alkyl para toluene sulfonates in

lamivudine at trace level concentration have been developed and validated as per ICH guidelines. The effectiveness of the two methods was ensured by the specificity, precision, accuracy and robustness. Hence, both the methods well suits for their intended purposes and can be successfully applied for the release testing of lamivudine into the market.

Acknowledgment

The authors are grateful to Dr. B. Parthasarathi Reddy, CMD, Hetero Group of Companies, Hyderabad, India for providing facilities to carry this research work.

References

- [1] European Medicines Agency, Guideline on the Limits of Genotoxic Impurities, CPMP/SWP/5199/02, EMEA/CHMP/QWP/251344/2006 (2007).

- [2] N.V.V.S.S. Raman, A.V.S.S. Prasad, K. Ratnakar Reddy, Strategies for the identification, control and determination of genotoxic impurities in drug substances: A pharmaceutical industry perspective, *J. Pharm. Biomed. Anal.* 55 (2011) 662–667.
- [3] A.V. Singh, L.K. Nath, N.R. Pani, Development and validation of analytical method for the estimation of lamivudine in rabbit plasma, *J. Pharm. Anal.* 1 (4) (2011) 251–257.
- [4] K. Ramakrishna, N.V.V.S.S. Raman, K.M.V. Narayana Rao, et al., Development and validation of GC–MS method for the determination of methyl methanesulfonate and ethyl methanesulfonate in imatinib mesylate, *J. Pharm. Biomed. Anal.* 46 (2008) 780–783.
- [5] I. Colon, S.M. Richoll, Determination of methyl and ethyl esters of methanesulfonic, benzenesulfonic and p-toluenesulfonic acids in active pharmaceutical ingredients by solid-phase microextraction (SPME) coupled to GC/SIM-MS, *J. Pharm. Biomed. Anal.* 39 (2005) 477–485.
- [6] R. Alzaga, R.W. Ryan, K.T. Worth, et al., A generic approach for the determination of residues of alkylating agents in active pharmaceutical ingredients by in situ derivatization headspace gas chromatography–mass spectrometry, *J. Pharm. Biomed. Anal.* 45 (2007) 472–479.
- [7] D.P. Elder, A. Teasdale, A.M. Lipczynski, Control and analysis of alkyl esters of alkyl and aryl sulfonic acids in novel active pharmaceutical ingredients (APIs), *J. Pharm. Biomed. Anal.* 46 (2008) 1–8.
- [8] G.E. Taylor, M. Gosling, A. Pearce, Low level determination of p-toluenesulfonate and benzenesulfonate esters in drug substance by high performance liquid chromatography/mass spectrometry, *J. Chromatogr. A* 1119 (2006) 231–237.
- [9] C.E. Rde, M.C. Salvadori, G. Suarez-Kurtz, A rapid and sensitive method for simultaneous determination of lamivudine and zidovudine in human serum by on-line solid-phase extraction coupled to liquid chromatography/tandem mass spectrometry detection, *Rapid Commun. Mass Spectrom* 18 (2004) 1147–1155.
- [10] Y. Alnouti, S.R. Lewis, C.A. White, et al., Simultaneous determination of zidovudine and lamivudine from rat tissues by liquid chromatography/tandem mass spectrometry, *Rapid Commun. Mass Spectrom.* 4 (2005) 503–508.
- [11] H.N. Mistri, A.G. Jangid, A. Pudage, et al., High throughput LC-MS/MS method for simultaneous quantification of lamivudine, stavudine and nevirapine in human plasma, *J. Chromatogr. B* 853 (2007) 320–322.
- [12] G. Bedse, V. Kumar, S. Singh, Study of forced decomposition behavior of lamivudine using LC, LC-MS/TOF and MSⁿ, *J. Pharm. Biomed. Anal.* 49 (2009) 55–63.
- [13] Z. Li, C. Ding, Q. Ge, et al., Simultaneous determination of lamivudine, stavudine and nevirapine in human plasma by LC-MS/MS and its application to pharmacokinetic study in clinic, *Biomed. Chromatogr.* 24 (2010) 926–934.
- [14] International Conference on Harmonisation Guideline on Validation of Analytical Procedures, Q2 (R1); 2005.