



Short communication

Novel bio analytical method development, validation and application for simultaneous determination of nebivolol and S-amlodipine in human plasma using ultra performance liquid chromatography-tandem mass spectrometry



Bhargav M. Patel^a, Arvind G. Jangid^b, B.N. Suhagia^c, Nirmal Desai^{a,*}

^a Department of Chemistry, St. Xavier's College, Navrangpura, Ahmedabad 380009, India

^b Accutest Research Lab (I) Pvt Ltd, India

^c Dharamsinh Desai University, Nadiad, Gujarat, India

ARTICLE INFO

Article history:

Received 20 August 2017

Received in revised form 3 January 2018

Accepted 4 January 2018

Available online 6 January 2018

Keywords:

S-amlodipine

Nebivolol

UPLC-MS/MS

ABSTRACT

A sensitive and specific ultra-performance liquid chromatography tandem mass spectrometric (UPLC-MS/MS) method has been developed, validated and applied for the assay of Nebivolol and S-amlodipine in human plasma. Sample extraction was carried out through hybrid extraction method from 250 μ L of human plasma sample. Linearity of the method was ($r \geq 0.9996$) was found to be dynamic for both the analytes over concentration range of 25.0–4000 pg/mL. Chromatographic separation was achieved on UPLC column {Waters Acquity UPLC BEH C18 (50 mm \times 2.1 mm, 1.7 micrometer)} with the mobile phase composition of 0.1% (v/v) formic acid in 5 mM Ammonium formate in water-acetonitrile (20:80, %v/v). Analytes Stability was assured under different requisite conditions in human plasma, reconstitution solution and diluents. Inter and intra-day assay precision and relative error (accuracy) were within $\pm 5\%$ for both analytes. The method was applied and reproduced to support a pharmacokinetic study of 5 mg Nebivolol (NEB) and 2.5 mg S-amlodipine (LAM) tablet on 9 healthy subjects.

© 2018 Elsevier B.V. All rights reserved.

1. Introduction

Nebivolol is highly selective β_1 -receptor blocker used for the treatment of hypertension and chronic heart failure. Nebivolol hydrochloride is chemically known as α, α' -[iminobis(methylene)] bis[6-fluoro-3,4-dihydro-2H-1-benzopyran-2-methanol] hydrochloride. NEB is third generation antihypertensive agent and has dual actions; it follows nitric oxide mediated pathway resulting in mild to moderate dilating blood vessels properties. In addition it also synergies the action of lowering blood pressure by reducing peripheral vascular resistance and increasing the stroke volume by maintaining the cardiac output [1]. NEB is metabolized by glucuronidation and hydroxylation by CYP2D6. The hydroxylated and glucuronidated metabolites are considered pharmacologically active, which contributes to an equally similar clinical profile for both CYP2D6 phenotypes [2,3]. Half life of NEB is considerably higher for the poor metabolizer people than extreme metabolizer

people. So there is potential need for individualized treatment strategies in order to manage nebivolol therapy [4].

Amlodipine, (RS) 3-ethyl 5-methyl-2-[(2-amino ethoxymethyl) – 4 – (2 – chlorophenyl) – 1,4 – dihydro-6-methyl-3,5-pyridinedicarboxylate, is a potent calcium channel blocker used in the treatment of angina and hypertension. “R” and “S” enantiomer do not have the same biological activity. Only S-amlodipine dilates the blood vessels and improves the blood flow [5].

NEB and LAM combination therapy is prescribed because they have complementary mechanisms of action which exerts symbiotic effect in lowering of blood pressure and more over this combination therapy allows lower dosage requirements of each drug. Punzi et al. [6] had reported that NEB can produce sustained reductions in blood pressure throughout more effectively than metoprolol in combination with S-amlodipine.

Literature revealed that, several methods have been reported for the determination of NEB and LAM individually or in combination with other drug in biological samples [7–14]. Ramakrishna et al. [7] and Nandania et al. [8] had proposed methods that included only analysis of nebivolol. Both the methods are rapid enough but less sensitive. Selvan et al. [9] analyzed nebivolol in combination

* Corresponding author.

E-mail address: nirmal.desai@sxca.edu.in (N. Desai).

with valsartan by LC–MS/MS. Ramakrishna et al. [7] and Selvan et al. [9] had used analogue internal standards while use of labeled isotopes as internal standard is prime requirement of regulatory bodies now a day. Ramakrishna et al. [7] had used higher injection volume (25 μ L) which may affect the reproducibility of method for long batch. Nandania et al. [8] had used higher (500 μ L) sample processing volume.

Several analytical methods have been developed to determine LAM in biological fluids, including HPLC, amperometric and GC detection [10–12]. These methods are good enough but with the limitation of sensitivity, higher retention time [10,11] and limited recovery [12]. Shentu et al. [13] reported method for the analysis of amlodipine by HPLC–tandem mass spectrometry with 12 min of total acquisition time. Rapid method was developed only for amlodipine with higher sample preparation volume of 0.5 mL by Ma et al. [14] using ultra performance liquid chromatography–tandem mass spectrometry.

SI Qian et al. [15] had also analyzed nebivolol by using amlodipine as an internal standard. However, till date there are no bioanalytical methods reported for the simultaneous determination of NEB and LAM in human plasma so that estimation of both drugs from single pill can be easily evaluated. So the aim of the present work was to develop and validate the method for the simultaneous estimation of NEB and LAM from single sample and apply the developed method in pharmacokinetic study. Here, we have applied method for the pharmacokinetic study to get the overview of the variation in PK parameters due to drug interaction.

2. Experimental

2.1. Chemicals and materials

Reference standard of Nebivolol (Purity 99.7%) and S-amlodipine (Purity 92.4%) were acquired from United States Pharmacopeia and Emcure Pharmaceuticals, respectively. Nebivolol D4 HCl (NED) (Purity 99.48%) and Amlodipine Maleate D4 (AMD) (Purity 99.86%) were procured from VIVAN life sciences Pvt. Ltd. (Mumbai, India). HPLC grade methanol and acetonitrile were obtained from J.T. Baker Inc. (Phillipsburg, NJ, USA). AR grade formic acid was procured from Qualigens Fine Chemicals (Mumbai, India). Ethyl acetate was purchased from Merck Specialties Pvt. Ltd. (Mumbai, India). Phenomenex StrataTM-X (30 mg, 1 cc) solid phase extraction cartridges were obtained from Phenomenex India (Hyderabad, India). Purified Milli-Q water was used from Millipore system (Bangalore, India). Blank human plasma with Na heparin as an anticoagulant was obtained from Supra tech Micro Path Laboratories (Gujarat, India) and was stored at $-20 \pm 5^\circ\text{C}$.

2.2. Liquid chromatographic and mass spectrometric conditions

Waters Acquity UPLC system (Massachusetts, USA) was used under reverse-phase conditions for chromatographic analysis. Desired peak separation was accomplished on Waters Acquity UPLC BEH C18 (50 mm \times 2.1 mm, 1.7 μ m) column by the mobile phase composition of 0.1% (v/v) formic acid in 5 mM Ammonium formate in water–acetonitrile (20:80, % v/v). Column oven and auto sampler temperature were set to $40 \pm 3^\circ\text{C}$ and $10 \pm 3^\circ\text{C}$, respectively. Isocratic flow rate of the mobile phase was constant at 0.510 mL/min. The total chromatographic run time was set to 2.1 min. Triple quadrupole mass spectrometer {Model: AB SCIEX API-4000 (Toronto, Canada)}, consisted with electro spray ionization mechanism in positive polarity mode was used for the quantitation of NEB, LAM and deuterated ISs. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor precursor \rightarrow product ion transitions for NEB (m/z

406.2 \rightarrow 151.1), LAM (m/z 409.1 \rightarrow 238.1), NED (m/z 410.2 \rightarrow 151.1) and AMD (m/z 413.1 \rightarrow 238.1). Analyst classic software version 1.5.2 was used to control all parameters of UPLC and MS/MS.

2.3. Calibrators and quality control samples

Nine calibration standards (CSs) were prepared for the concentration level 25.0, 50.0, 100, 400, 800, 1600, 2400, 3400 and 4000 pg/mL for NEB and LAM. Four different QC samples were prepared at concentration, (1) 25.0 pg/mL (LLOQ QC, lower limit of quantification quality control), (2) 70.0 pg/mL (LQC, low quality control), (3) 1250 pg/mL (MQC, medium quality control), and (4) 3200 pg/mL (HQC, high quality control) for both analyte. Details for the preparation of stock solutions and their dilutions are covered in Appendix A–Supplementary data.

2.4. Plasma sample extraction

To an aliquot of 250 μ L of spiked plasma sample, 50 μ L of combined internal standard working solution of NED and AMD (1250 pg/mL) was added and vortexed for 5 s. Then 150 μ L of 1% (v/v) Formic acid in water was added as an extraction buffer and mixed for 10 s. Thereafter, analytes in sample was extracted by 2.0 mL ethyl acetate on rotor for 10 min at $18 \times g$. The organic layer was then separated by centrifuging the sample at $1431 \times g$ for 5 min at 10°C . The supernatant organic layer was then separated and evaporated to dryness in a temperature controlled water-bath maintained at 50°C under a gentle stream of nitrogen gas. After drying, the residue was reconstituted by 500 μ L of methanol. Subsequently, after brief vortex, extracted sample was loaded to Phenomenex Strata-X (30 mg, 1 cc) cartridges, which were pre-conditioned with 1 mL methanol followed by 1 mL of 1% (v/v) Formic acid in water. Each sample–cartridge was washed with 1.0 mL, 10% (v/v) methanol in water. The analytes and ISs were finally eluted with 300 μ L of mobile phase in to pre-labeled vials and vortexed for 15 s. 5 μ L of final sample was used for injection in full loop injection mode. “Load ahead” function was implemented to control overall analysis time.

2.5. Matrix assessment and other validation parameter

As per regulatory guidance requirements [16], a detailed evaluation of matrix effect is prerequisite condition for bioanalytical assays validated through LC–MS/MS [17]. Generally, matrix effect attributes to either suppression or enhancement of ionization efficiency of an analyte resulting from the presence of co-extracted endogenous or exogenous components. All validation parameters were determined based on USFDA guidelines [16] and described in Appendix A–Supplementary data.

2.6. Pharmacokinetic study & incurred sample reanalysis

The Pharmacokinetic interaction study was performed by two reference products. Nebilet (Nebivolol), 5 mg tablet of Biolab Sanus Farmacêutica Ltda, Brazil and Novanlo (Levoamlodipine besilate), 2.5 mg tablet of Biolab Sanus Farmacêutica Ltda, Brazil. The study was conducted as per International Conference on Harmonization, E6 Good Clinical Practice guidelines. Total 36 subject samples were analyzed successfully to check the reproducibility of assay by incurred sample reanalysis experiment. Details are described in Appendix A–Supplementary data.

3. Results and discussion

3.1. Method development

3.1.1. Optimization of mass spectrometric condition and liquid chromatographic condition

It is of paramount significance to optimize mass parameters and chromatographic conditions to develop and validate sensitive, rugged and rapid method for the real-time determination of Nebivolol and S-amlo地平ine in human plasma. Mass parameters were tuned with electrospray ionization source in positive as well as negative ionization mode by using 250 ng/mL of tuning solution. NEB, LAM and their internal standards are basic in nature containing secondary amine functionality which can be easily protonated consequently signal intensities in positive polarity mode were much higher than those in negative ion mode. Nebivolol, S-amlo地平ine, Nebivolol D4 and Amlodipine D4 gave pre-dominant protonated ($M + H$)⁺ parent ions at m/z 406.2, 409.1, 410.2 and 413.1 ions, respectively in Q1 MS full scan spectra. Collision energy 40 V and 20 V was sufficient to break the parent ions of NEB and LAM, respectively. The most abundant ions found in the product ion mass spectrum were m/z 151.1 and 238.1 for NEB and LAM, respectively. Fragmentation pattern of NEB and LAM was in accordance with the report published by J. Nandania et al. [8] and J. Shentu et al. [13], respectively. Declustering potential and collision energy were confirmed by observing the maximum presence of the product ion. The ion source temperature was suitably set at 400 °C while ion spray voltage was optimized at 2500 V for maximum ionization efficiency.

The chromatographic conditions were evaluated to accomplish an efficient separation, symmetric peak shapes and resolution of NEB and LAM as both the drugs have marginal divergence in physico-chemical properties. Apart from selective elution of analytes, emphasis was also given to sensitivity and high throughput. This was achieved by wide array of column and combinations of mobile-phase. Different volume ratios of acetonitrile–aqueous combinations were optimized as mobile phase, along with formic acid, ammonium formate and ammonium tri fluoro acetate buffers with different strength on ACQUITY UPLC HSS C18 (50 mm × 2.1 mm, 1.8 μ m) and Pursuit XRs Ultra C18 (50 mm × 2.0 mm), 2.8 μ m columns. Optimal resolution was obtained in mobile phase composition of acetonitrile–0.1% (v/v) formic acid in ammonium formate in water (5 mM) (80:20, v/v) but it could not harmonize with the accepted chromatography with ACQUITY UPLC HSS C18 (50 mm × 2.1 mm, 1.8 μ m) column. This mobile phase shows higher base line for LAM with Pursuit XRs Ultra C18 (50 mm × 2.0 mm), 2.8 μ m column and which lead to the specificity issues. The use of UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μ m) chromatographic column offered superior peak shape, baseline separation, desired linearity and reproducibility for both compounds in a very short run time of 2.1 min for each run by 0.510 mL/min of mobile phase [acetonitrile–0.1% (v/v) Formic acid in ammonium formate in water (5 mM) (80:20, v/v)] flow rate.

3.1.2. Plasma extraction

In our endeavour to procure cleaner plasma samples with prominent signal and pertinent recovery of both the analytes and ISs, several sample preparation experiments were carried out with three different extraction procedures. To achieve this aim it was crucial to have an efficient and limited steps extraction procedure as this also ensures maximum elimination of phospholipids in plasma sample. Protein precipitation was chosen initially to have a simple and single step extraction procedure. Methanol, acetone and acetonitrile were used as precipitating solvents. The extracts were clear from plasma content but the recovery of LAM was very poor for all the solvents. Addition of formic acid to these solvents

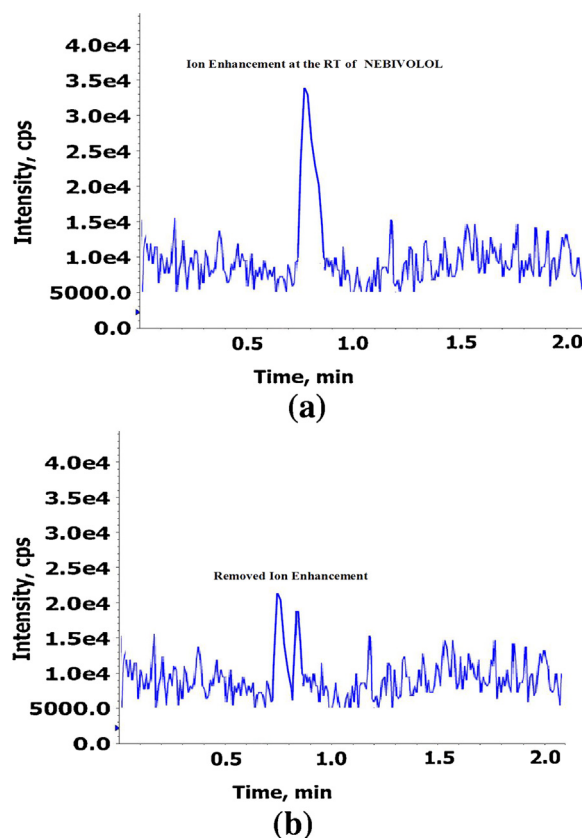


Fig 1. Matrix effect profiles obtained by post-column infusion of Nebivolol through (a) Liquid-liquid extraction with ethyl acetate (b) Solid phase extraction with Phenomenex Strata™-X (30 mg, 1 cc) cartridges including sample washing step of 10% (v/v) methanol in water.

in different percentage volume/volume ratio was unsuccessful in obtaining persistent and reproducible response. Liquid-liquid extraction was tested using different ether solvents like methyl *tert*-butyl ether and diethyl ether separately as suggested by Y. Ma et al. [15] and in combination with dichloromethane as reported by N.V.S. Ramakrishna et al. [7] and J. Nandania et al. [8]. It failed to achieve matrix free samples for both analytes. Ethyl acetate was also implemented as extraction solvent. Acidic and alkaline conditions were entrenched by diluted solutions of sodium hydroxide, formic acid and hydrochloric acid to cleave the drug-protein binding. From all experimental trials, it was concluded that liquid-liquid extraction (LLE) method using ethyl acetate as organic solvent in presence of 150 μ L of 1.0% (v/v) formic acid in water followed by the reconstitution with 500 μ L methanol shown better results in terms of signal, separation and peak shape. But it failed to prove the method specificity for NEB. The presence of phospholipids at the analytes retention time was confirmed by post column infusion experiment of particular sample for NEB which finally resulted in ion-enhancement (Fig. 1a). From these results, it was concluded that there is a necessity to extract more clear samples by removing lysophospholipids and glycerophospholipids.

Finally, as per our experience, Phenomenex Strata™-X (30 mg, 1 cc) extraction cartridges plays better role for the removal of phospholipids [18]. Above extracted samples were then loaded on Phenomenex Strata™-X (30 mg, 1 cc) cartridges, which were pre-conditioned with 1 mL methanol followed by 1 mL of 1% (v/v) Formic acid in water. Washing of each cartridge with 1 mL, 10% (v/v) methanol in water also becomes vital step for the removal of phospholipids. Analytes and ISs were finally eluted by 300 μ L of mobile phase (Fig. 1b).

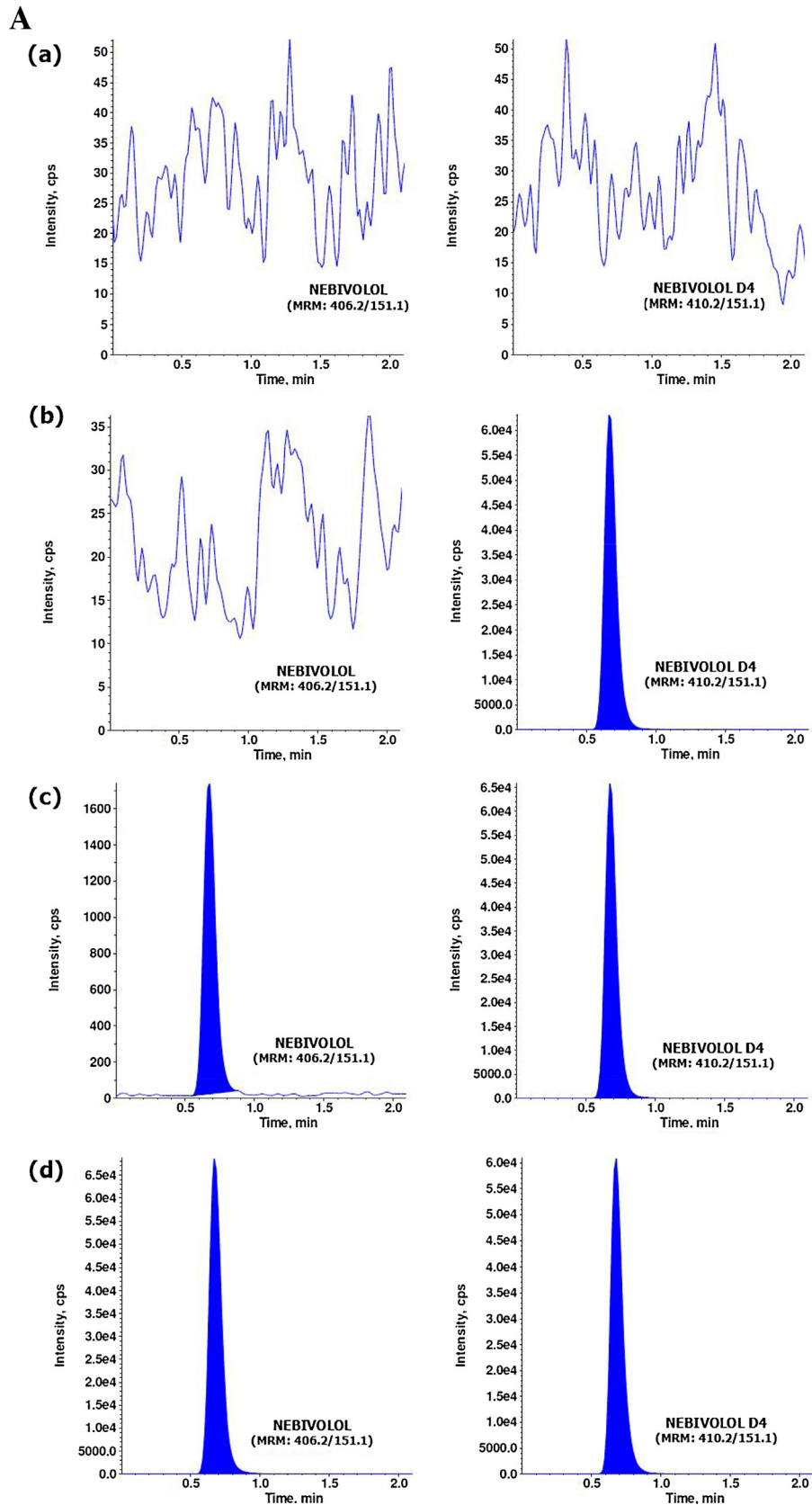


Fig. 2. (A) MRM ion-chromatograms of (a) double blank plasma, without nebivolol and its IS, (b) blank plasma with IS (c) Nebivolol at LLOQ, (d) Nebivolol in subject sample at C_{max} after oral administration of 5 mg Nebivolol and/or 2.5 mg S-amlodipine tablet. (B) MRM ion-chromatograms of (p) double blank plasma, without S-amlodipine and IS, (q) blank plasma with IS, (r) S-amlodipine at LLOQ, (s) S-amlodipine in subject sample at C_{max} after oral administration of 2.5 mg S-amlodipine and/or 5 mg Nebivolol tablet.

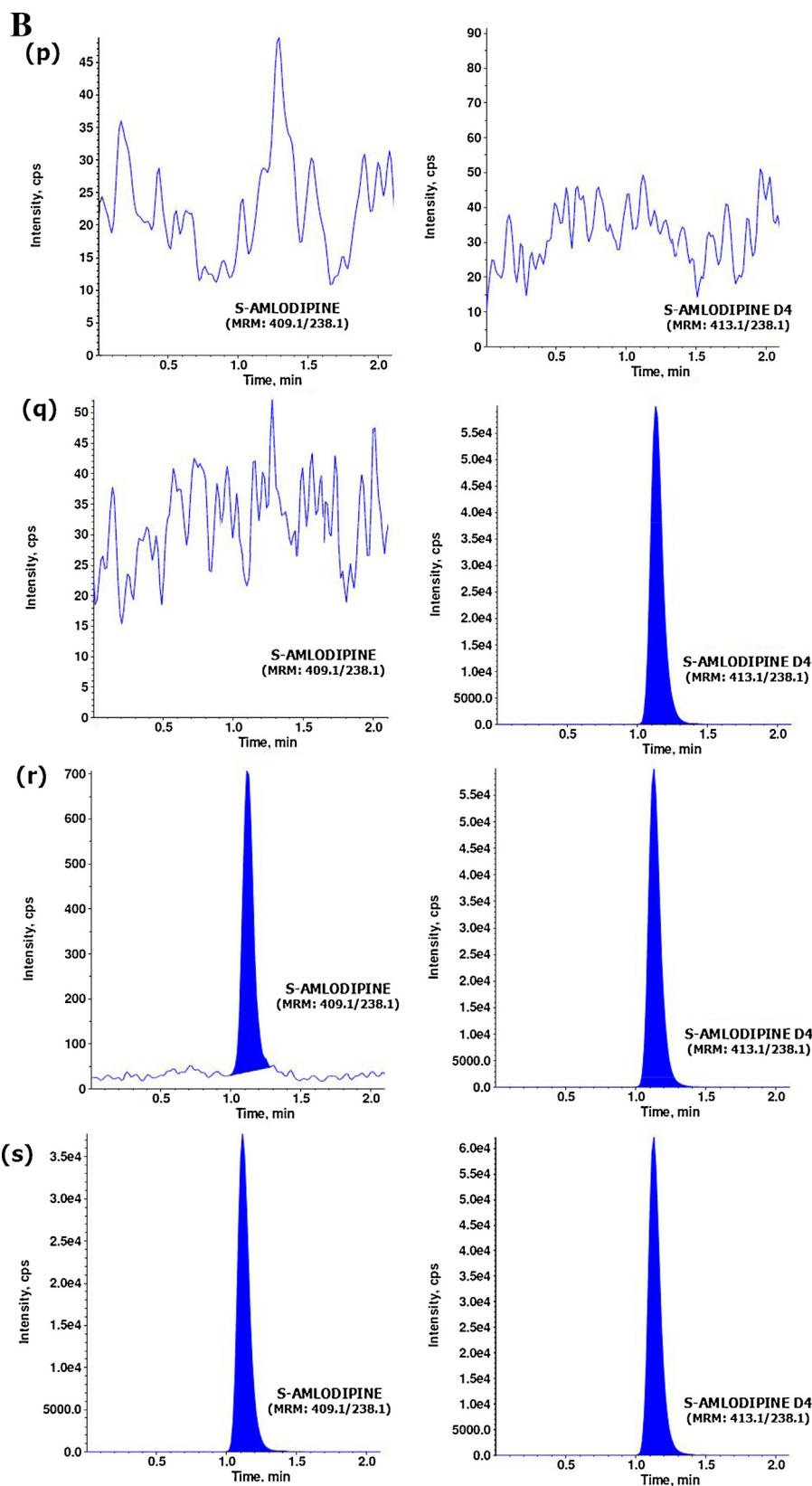


Fig. 2. (Continued)

Finally we have imply LLE followed by solid phase extraction (SPE) technique as nebivolol was not uniformly extracted from plasma samples (directly) by SPE cartridge and literature also supports LLE methodology for the extraction of nebivolol.

3.2. Method validation results

System suitability, system performance, and carry-over data demonstrated adequate assay performance as shown in Supple-

Table 1

Internal Standard Normalized Matrix Factor at LQC and HQC level for different ten plasma lots.

Sr. No.	Sample id in difference plasma lots	Internal Standard Normalized Matrix Factor for nebivolol		Internal Standard Normalized Matrix Factor for S-amlodipine	
		LQC	HQC	LQC	HQC
1	Plasma Lot -1	1.013	1.024	1.014	1.053
2	Plasma Lot -2	1.007	1.007	1.022	0.995
3	Plasma Lot -3	1.020	1.020	0.998	1.028
4	Plasma Lot -4	1.002	1.049	1.028	0.993
5	Plasma Lot -5	1.034	1.022	0.957	1.058
6	Plasma Lot -6	1.029	1.073	0.995	1.023
7	Plasma Lot-7 (2% hemolysed)	1.021	1.039	0.997	1.076
8	Plasma Lot-8 (5% hemolysed)	1.030	1.020	1.011	1.047
9	Plasma Lot-9 (250 mg/dL Lipemic)	1.048	0.983	1.039	1.038
10	Plasma Lot-10 (300 mg/dL Lipemic)	1.032	1.017	1.047	1.031
	MEAN	1.024	1.025	1.011	1.034
	SD	0.014	0.024	0.026	0.026
	% CV	1.37	2.34	2.57	2.51

Table 2

Extraction recovery for Nebivolol and S-Amlodipine.

QC ID	Analyte	A (% CV)	B (% CV)	Extraction recovery (% RE) [A/B × 100]
LQC	Nebivolol	49192 (2.20)	56091 (1.08)	87.7 (88.1) ^h
	S-Amlodipine	25847 (1.73)	29950 (1.98)	86.3 (85.9) ⁱ
MQC	Nebivolol	950587 (2.53)	1080213 (2.64)	88.0 (86.9) ^h
	S-Amlodipine	436678 (3.59)	519237 (4.42)	84.1 (85.2) ⁱ
HQC	Nebivolol	2368651 (2.35)	2799824 (0.88)	84.6 (85.9) ^h
	S-Amlodipine	1444631 (1.11)	1619541 (1.64)	89.2 (87.3) ⁱ

A: Mean area response of six replicate samples prepared by spiking before extraction.

B: Mean area response of six replicate samples prepared by spiking in extracted blank plasma.

CV: coefficient of variation.

^h Values for internal standard, Nebivolol D4.ⁱ Values for internal standard, Amlodipine D4.

mentary Table S1. Results of the precision (% CV) and accuracy values on different column and different analyst for method ruggedness were ranged from 1.46 to 6.35% and 96.24–105.30%, respectively across all QC levels. Dilution reliability with 1/4th and 1/10th dilution was also accepted in the range mentioned in Supplementary Table S1.

The selectivity of the method has been represented by chromatogram (Fig. 2) of double blank plasma, blank plasma (spiked with IS), analytes at LLOQ and in subject sample at C_{max} concentration, it also demonstrates that there was no significant impact of any phospholipids or endogenous compound observed from double blank plasma at the retention time of NEB, LAM and ISs during the bio analytical method validation thoroughly. In addition, none of the commonly used medications by human volunteers interfered at RT of analytes of interest.

As presence of unmonitored, co-eluting compounds from the matrix can directly impact the overall reliability of a method, it is recommended to evaluate matrix factor (MF) to consider the matrix effect. Descriptive results of matrix effect are illustrated in Table 1. The IS-normalized MF obtained from 0.983–1.073 and 0.957–1.076 for NEB and LAM, respectively. For relative matrix effect and mean of the slope of the ten different calibration curves were 0.00123 and 0.00120 for NEB and LAM respectively, whereas %CV was 1.02 and 1.63 for NEB and LAM respectively, which was well within admissible limits. Furthermore, the extracts obtained through SPE showed negligible matrix effect, when analyzed by results of the post column analyte infusion experiment which supports the matrix effect free assay. The mean extraction recovery ranged from 84.6–88.0% for NEB and 86.3–89.2% for LAM at all QC levels. Detailed data of extraction recovery are presented in Table 2.

The calibration curves showed acceptable linearity ($r \geq 0.9996$) in the established concentration range of 25.0–4000 pg/mL for each drug. The mean linear equation for concentrations of

calibration curve standards was $y = (0.00124 \pm 0.00008) x + (-0.00183 \pm 0.00039)$ for NEB and $y = (0.00120 \pm 0.00005) x + (-0.00160 \pm 0.00030)$ for LAM. The accuracy (%) and precision (% CV) values for calibration standards were found to be from 94.91 to 106.63% and 0.99–3.08% for NEB and 95.15–104.74% and 1.03–4.12% for LAM respectively. The lower limit of detection (0.50 ng/mL) was measured at a signal-to-noise ratio (S/N) ≥ 21 for both the analytes. The intra-batch and inter batch precision (% CV) varied from 1.54–4.32% and the accuracy was well within 96.03–104.07% for NEB and LAM (Supplementary Table S2).

The short-term and long-term stock solutions stability of analytes and ISs established for 18 h and for 06 days respectively with a change of ≤ 2.4 . The detailed results of analytes stability in plasma are given in Table 3.

3.3. Application of the method in human subjects and incurred sample reanalysis

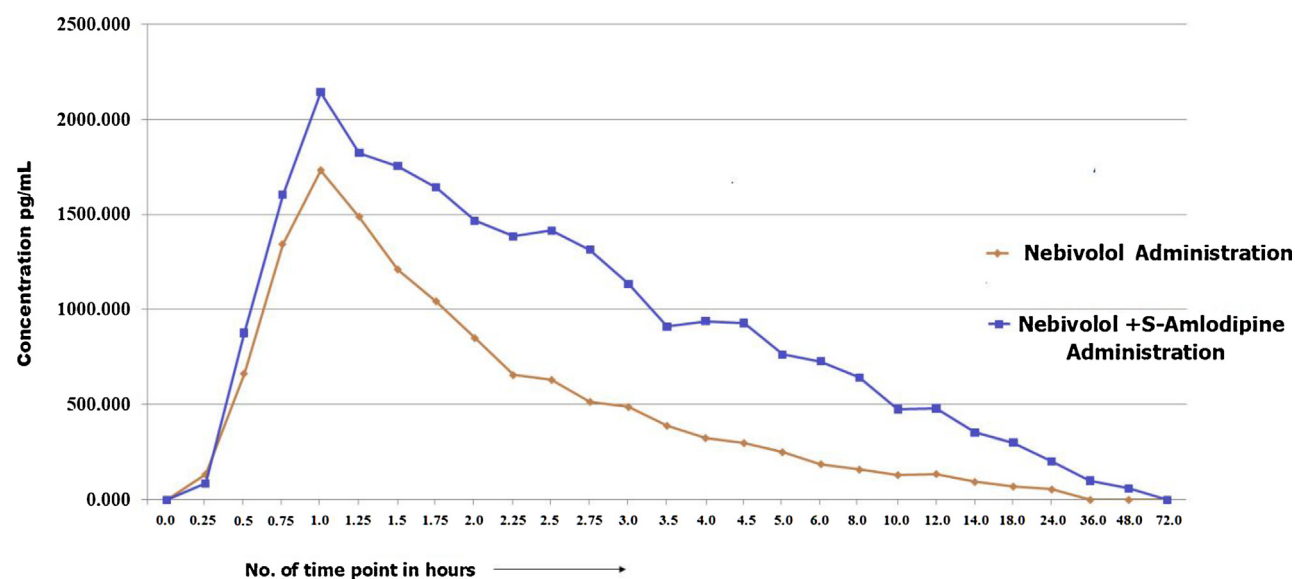
A study was designed with 9 healthy subjects through oral administration of 5 mg nebivolol and/or 2.5 mg S-amlodipine tablets. Fig. 3 shows the plasma concentration vs. time profile of NEB and LAM in healthy volunteers under fasting condition. Supplementary Table S3 summarizes the pharmacokinetic parameters viz. C_{max}, AUC_{0–t}, AUC_{0–∞}, T_{max}, K_{el} and t_{1/2} which were calculated for NEB and LAM after oral administration of reference A (Nebilet; 5 mg Nebivolol of Biolab Sanus Farmacêutica Ltda, Brazil), reference B (Novanlo; 2.5 mg Levoamlodipine besilate of Biolab Sanus Farmacêutica Ltda, Brazil) and reference C (combination of reference A+ reference B). Here, in application of the method our aim was to get the overview of the variation in PK parameters because of the inter action of drugs. From the Supplementary Table S3 we can conclude that almost same results for each parameter were found for alone and in combination therapy for each drug.

Table 3
Stability results for Nebivolol and S-Amlodipine under different conditions (n = 6).

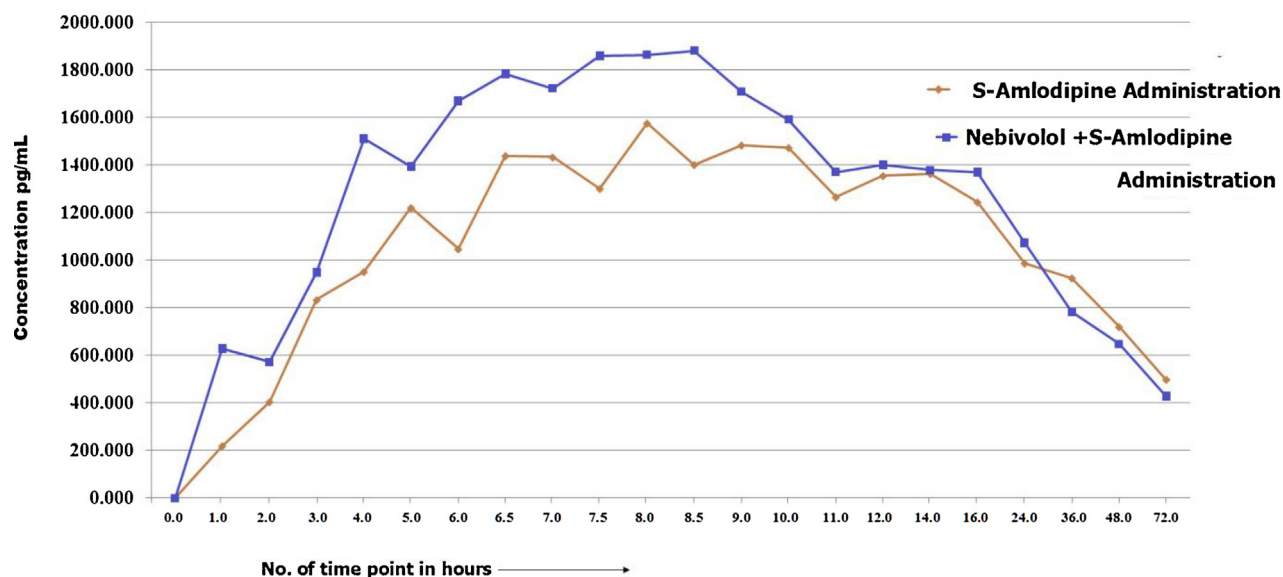
Stability	Storage condition	Nebivolol			S-Amlodipine		
		Standard conc.(pg/mL)	Mean calculated conc.(pg/mL) ± SD	% Mean change	Standard conc.(pg/mL)	Mean calculated conc.(pg/mL) ± SD	% Mean change
Bench top	5 h at 25 ± 5 °C (Room temperature)	70.0	68.383 ± 0.316	−2.31	70.0	72.786 ± 0.142	3.98
		3200.0	3250.88 ± 7.730	1.59	3200.0	3251.52 ± 2.897	1.61
Extracted sample stability	Auto sampler (6 °C, 52 h)	70.0	69.237 ± 0.166	−1.09	70.0	68.194 ± 0.349	−2.58
		3200.0	3094.08 ± 5.729	−3.31	3200.0	3394.56 ± 6.079	6.08
Freeze and thaw	3 h at 25 ± 5 °C (Room temperature)	70.0	71.442 ± 0.287	2.06	70.0	69.314 ± 0.267	−0.98
		3200.0	3124.16 ± 4.693	−2.37	3200.0	3151.04 ± 2.630	−1.53
Dry extract stability	After 7 th cycle at −20 °C	70.0	65.758 ± 0.756	−6.06	70.0	67.557 ± 0.401	−3.49
		3200.0	2914.88 ± 6.064	−8.91	3200.0	2992.32 ± 3.193	−6.49
Stability in matrix	47 h at 5 ± 3 °C	70.0	72.603 ± 1.322	3.72	70.0	74.603 ± 3.121	6.58
		3200.0	3206.646 ± 1.805	0.21	3200.0	3221.535 ± 9.754	0.67
Stability in matrix	32 days at −20 ± 5 °C	70.0	68.131 ± 0.593	−2.67	70.0	69.272 ± 0.571	−1.04
		3200.0	3102.40 ± 8.200	−3.05	3200.0	3275.20 ± 5.151	2.35
		70.0	68.614 ± 0.467	−1.98	70.0	71.148 ± 0.268	1.64
	32 days at −70 ± 10 °C	3200.0	3282.56 ± 4.767	2.58	3200.0	3310.72 ± 3.765	3.46

SD: standard deviation.

% mean change = (mean stability samples – mean comparison samples/mean comparison samples) × 100.



a. Nebivolol Profile



b. S-Amlodipine Profile

Fig. 3. Pharmacokinetic profile of Nebivolol and S-amlodipine of a particular subject after oral administration of 5 mg Nebivolol and/or 2.5 mg S-amlodipine tablet.

The 90% confidence intervals of the mean ratio (test/reference) of In-transformed data were within the acceptance range of 80% to 125% for AUC_{0-t} and AUC_{0-inf} and within the acceptance range for C_{max} . Further there was no adverse event observed during study. About 1004 samples including the calibration and QC samples along with subject samples were analyzed within 3 days. Furthermore, the assay reproducibility of 36 incurred sample reanalysis showed $\pm 9\%$ change in the results with respect to initial test results. This acknowledges the reproducibility of the proposed validated method (Supplementary Fig. S1).

4. Conclusions

To the best of our knowledge this is first report on the simultaneous estimation of NEB and LAM in human plasma by UPLC–MS/MS. Assay performed by the presented work was found to be rapid, novel, sensitive, selective and matrix effect free. Results of the incurred sample reanalysis experiment in acceptable range confirms the reproducibility of the method. The sensitivity of the proposed method is adequate enough to support a wide range of pharmacokinetic studies involving simultaneous estimation of NEB and LAM which can cover different types of subject samples on large scale.

Acknowledgement

The authors gratefully acknowledge Accutest Research Lab for providing necessary facilities to carry out this work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jpba.2018.01.004>.

References

- [1] V. C. O. Kamp, G.T. Sieswerda, Comparison of effects on systolic and diastolic left ventricular function of nebivolol versus atenolol in patients with uncomplicated essential hypertension, *Am. J. Cardiol.* 92 (2003) 344–348.
- [2] C. Lindamood, S. Ortiz, A. Shaw, R. Rackley, J.C. Gorski, Effects of commonly administered agents and genetics on nebivolol pharmacokinetics: drug–drug interaction studies, *J. Clin. Pharmacol.* 51 (2011) 575–585.
- [3] J. Fongemie, E. Felix-Getzik, A review of nebivolol pharmacology and clinical evidence, *Drugs* 75 (2015) 1349–1371.
- [4] C. Briciu, M. Neag, D. Muntean, C. Bocsan, A. Buzoianu, O. Antonescu, A.-M. Gheldiu, M. Achim, A. Popa, L. Vlase, Phenotypic differences in nebivolol metabolism and bioavailability in healthy volunteers, *Clujul Med.* 88 (2015) 208–213.
- [5] S. Goldmann, J. Stoltefuss, L. Born, Determination of the absolute configuration of the active amlodipine enantiomer as (–)-S: a correction, *J. Med. Chem.* 35 (1992) 3341–3344.
- [6] H.A. Punzi, Combination therapy with nebivolol/amlodipine is superior to metoprolol/amlodipine in the control of cuff and 24-hr ambulatory blood pressure, *J. Am. Soc. Hypertens* 9 (2015).
- [7] N.V.S. Ramakrishna, K.N. Vishwottam, M. Koteswara, S. Manoj, M. Santosh, D.P. Varma, Rapid quantification of nebivolol in human plasma by liquid chromatography coupled with electrospray ionization tandem mass spectrometry, *J. Pharm. Biomed. Anal.* 39 (2005) 1006–1013.
- [8] J. Nandania, S.J. Rajput, P. Contractor, P. Vasava, B. Solanki, M. Vohra, Quantitative determination of nebivolol from human plasma using liquid chromatography–tandem mass spectrometry, *J. Chromatogr. B* 923 (2013) 110–119.
- [9] P.S. Selvan, K.V. Gowda, U. Mandal, W.D.S. Solomon, T.K. Pal, Simultaneous determination of fixed dose combination of nebivolol and valsartan in human plasma by liquid chromatographic–tandem mass spectrometry and its application to pharmacokinetic study, *J. Chromatogr. B* 858 (2007) 143–150.
- [10] M. Josefsson, B. Norlander, Coupled-column chromatography on a Chiral-AGP phase for determination of amlodipine enantiomers in human plasma: an HPLC assay with electrochemical detection, *J. Pharm. Biomed. Anal.* 15 (1996) 267–277.
- [11] M. Josefsson, A.-L. Zackrisson, B. Norlander, Sensitive high-performance liquid chromatographic analysis of amlodipine in human plasma with amperometric detection and a single-step solid-phase sample preparation, *J. Chromatogr. B Biomed. Sci. Appl.* 672 (1995) 310–313.
- [12] A.P. Beresford, P.V. Macrae, D.A. Stopher, B.A. Wood, Analysis of amlodipine in human plasma by gas chromatography, *J. Chromatogr. B Biomed. Sci. Appl.* 420 (1987) 178–183.
- [13] J. Shentu, L. Fu, H. Zhou, X.J. Hu, J. Liu, J. Chen, G. Wu, Determination of amlodipine in human plasma using automated online solid-phase extraction HPLC–tandem mass spectrometry: application to a bioequivalence study of Chinese volunteers, *J. Pharm. Biomed. Anal.* 70 (2012) 614–618.
- [14] Y. Ma, F. Qin, X. Sun, X. Lu, F. Li, Determination and pharmacokinetic study of amlodipine in human plasma by ultra performance liquid chromatography–electrospray ionization mass spectrometry, *J. Pharm. Biomed. Anal.* 43 (2007) 1540–1545.
- [15] L. X. Qian Si, Yuan-cheng Chen, Li-hua Huang, Yu Cheng, Hua He, Determination of nebivolol in human plasma by LC–MS/MS and study of its pharmacokinetics on the Chinese, *J. China Pharma. Univ.* 42 (2011) 136–140.
- [16] Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM), 2001 (May).
- [17] A. Bergeron, F. Garofolo, Importance of matrix effects in LC–MS/MS bioanalysis, *Bioanalysis* 5 (2013) 2331–2332.
- [18] B. Patel, B.N. Suhagia, A.G. Jangid, H.N. Mistri, N. Desai, Systematic evaluation of matrix effect and cross-talk-free method for simultaneous determination of zolmitriptan and N-desmethyl zolmitriptan in human plasma: a sensitive LC–MS/MS method validation and its application to a clinical pharmacokinetic study, *Biomed. Chromatogr.* 30 (2016) 447–458.