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Quantification of Nebivolol in Human Plasma Using Stable Labeled Internal Standard by ESI-LC-MS/MS

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ABSTRACT

A rapid and sensitive LC-MS/MS method for the quantification of nebivolol using d4- nebivolol as internal standard has been developed and validated. The nebivolol and d4- nebivolol were extracted by liquid- liquid extraction using tert-butyl ethyl ether and separated on Kromasil 100-5C 4.6x100mm column using a mixture of 0.1% formic acid in 5 mM ammonium acetate, methanol and acetonitrile at composition of (20:20:60 v/v) at a flow rate of 0.5 mL/min. Detection involved an API-4000 LC-MS/MS with electrospray ionization in the positive mode. The method was validated as per the FDA guidelines and shown to provide an intra and inter day precision and accuracy within the acceptable limit with in a run time of 3.0 min. The proposed method can adopt for the regular bioequivalence study analysis and also can easily adoptable for clinical drug monitoring due to its simplicity and ruggedness.

Keywords: Nebivolol, Human plasma, Liquid–liquid extraction, LC-MS/MS.

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INTRODUCTION

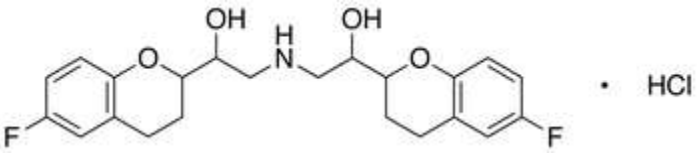
Hypertension affects more than seventy two million people in the United States and more than one billion people worldwide[1].The β-blocker class of medication is one of the oldest antihypertensive agents with a proven efficacy on hypertension and preventing cardio vascular diseases[2]. The usage of traditional β blockers is also associated with few adverse events such as fatigue, bradycardia, asthma exacerbation and impotence[3]. Nebivolol is a racemic combination of d-nebivolol [+SRRR] and l-nebivolol [-RSSS][4] belongs to third generation selective β blocker with a vasodilator effect5 . A recent study suggests nebivolol is a cardioselective β blocker and reported its more efficacy and well tolerability in patients with hypertension. Also, evidence that it has the potential to reduce mortality in patients with cardiac heart failure[6]. Numerous analytical methods have been reported in literature for the determination of nebivolol in formulations either as single or in combination with other drugs using high performance liquid chromatography on achiral[7-11] and chiral column[12-13]. A few bioanalytical methods were also reported for the determination of nebivolol in human plasma including radio immune assays[14], HPLC-fluorescence detection [15-16] and liquid chromatographic tandem mass spectrometry (LC-MS/MS) [17-21]. The proposed method can be comparable with the reported methods by Ramakrishna *et al*. [18], senthamil *et al*. [19] and jastin *et al.* [20]. But, in the proposed method few of the limitations of earlier methodologies have been conquered like high plasma volume, higher flow rates and the longer run time. Moreover, Ramakrishna *et al*. [18] described the advantages of using labeled internal standard where they were not able to include in their methodology and also recommends the use of labeled internal standard for the bioanalytical method. The author considered all the literature implications and developed and validated high throughput LC-MS/MS bioanalytical method for the determination of nebivolol in human plasma. At a glance, the proposed method is very rugged and validated as per the current regulatory guidelines. It can be adoptable for the regular bioequivalence studies and also be taken-up for the clinical drug monitoring.

MATERIALS AND METHOD

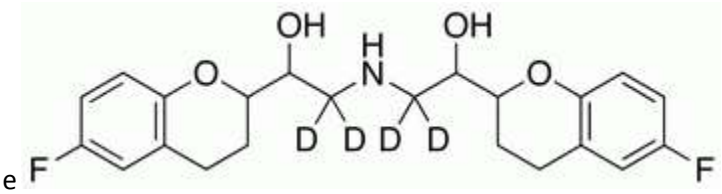
Chemicals and Materials

The reference standards of nebivolol hydrochloride (99.25%) and Nebivolol d4 (99.34%) were obtained from Hetero Drugs Limited (Hyderabad, India) and Vivan Life Sciences Limited (Mumbai, India), respectively. Their chemical structures are shown in Figure. 1. HPLC grade methanol, acetonitrile and *tert* butyl methyl ether were purchased from JT Baker (Phillipsburg, USA). Analytical grade formic acid, sodium hydrogen carbonate, ammonium acetate were

purchased from Merck Ltd (Mumbai, India). Water used for the LC–MS/MS analysis was prepared by using Milli Q water purification system procured from Millipore (Bangalore, India). The control human plasma sample was procured from Deccan's Pathological Lab's (Hyderabad, India).



(a)



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(b)

Figure 1: Chemical structures of (a) Nebivolol HCl and (b) Nebivolol d4

Instrumentation and Chromatographic conditions

An HPLC system (Shimadzu, Kyoto, Japan) consisting of a Kromasil 100–5C_{18} column (100 mm × 4.6 mm, 5 µm), a binary LC–20AD prominence pump, an auto sampler (SIL–HTc) and a solvent degasser (DGU–20A_3) was used for the study. Aliquot of 10 µL of the extracted samples were injected onto the column, which was kept at 40 ºC. An isocratic mobile phase consisting of a mixture of mobile phase buffer (0.1% formic acid in 5mM ammonium acetate), methanol and acetonitrile (20:20:60, v/v/v) was used to separate the analyte from the endoge*n*ous components and delivered at a flow rate of 0.5 mL/min into the electrospray ionization chamber of the mass spectrometer. Quantification was achieved with MS–MS detection in positive ion mode for the analyte and the IS using an MDS Sciex API–4000 mass spectrometer (Foster City, CA, USA) equipped with a Turboionspray ™ interface at 500 ºC. The ion spray voltage was set at 4500 V. The source parameters viz. the nebulizer gas (GS1), auxiliary gas (GS2), curtain gas and collision gas were set at 40, 30, 40, and 6 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were 95, 42, 10, 8 V for both nebivolol and nebivolol d4 (IS). Detection of the ions was carried out in the multiple–reaction monitoring mode (MRM), by monitoring the transition pairs of *m/z* 406.1 precursor ion to the *m/z* 151.1 product ion for nebivolol and *m/z* 410.1 precursor ion to the *m/z* 151.1 product ion for nebivolol d4. Both Q1 and Q3 Quadrupoles were set on unit resolution. The results obtained were processed by Analyst Software™ (version 1.6.1).

Preparation of plasma standards and quality controls

Standard stock solution of nebivolol and internal standard (1 mg/mL) were prepared in methanol. Working solutions for calibration and controls were prepared by appropriate dilution in methanol and water (60:40, v/v; diluent). The internal standard working solution (1µg/mL) was prepared by diluting its stock solution with diluent. Stock solutions of nebivolol and internal standard were found to be stable for 10 days at 2–8 °C. Calibration samples of nebivolol were prepared at a concentration levels of 0.051, 0.103, 0.206, 0.411, 1.028, 2.056, 4.112, 6.137, 8.182 and 10.228 ng/mL as a single batch at each concentration. Similarly, quality control (QC) samples were also prepared as a bulk based on an independent weighing of standard drug, at concentrations of 0.054 (LLOQ), 0.135 (low), 1.284 (middle 1), 5.137 (middle 2) and 7.725 ng/mL (high) as a single batch at each concentration. All plasma samples were stored in the freezer at –70 ± 10ºC until analyses.

Sample processing

The thawed samples were vortexed for 10 s prior to spiking. A 100 µL aliquot of human plasma sample was pipette into 15mL glass stoppered tubes, 50 µL of 1µg/mL d4-nebivolol dilution was added, except in blank plasma samples where 50 µL diluent was added. Then 50 µL of 50mM sodium hydrogen carbonate buffer was added and vortexed. 3 mL of *tert* butyl methyl ether (TBME) was added and shaken for 20 min on reciprocating shaker(Scigenics Biotech, Chennai, India) at 200 rpm and then centrifuged for 5 min at 4000 rpm on Megafuse 3SR (Heraeus, Germany). Samples were flash frozen using dry ice/methanol; the supernatant layer was transferred to a new set of pre-labeled 5 mL glass test tubes and the contents of the tubes evaporated in a stream of nitrogen at 40^{o}C. The dried extract was reconstituted with 200 µL of the mobile phase and a 10 µL aliquot of it was injected onto the LC–MS/MS system.

Bioanalytical method validation

The validation of the presented method was carried out as per US FDA guidelines (US DHHS, et al., 2001)[22]. The parameters determined were selectivity, specificity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity and stability.

RESULTS AND DISCUSSION

Method development

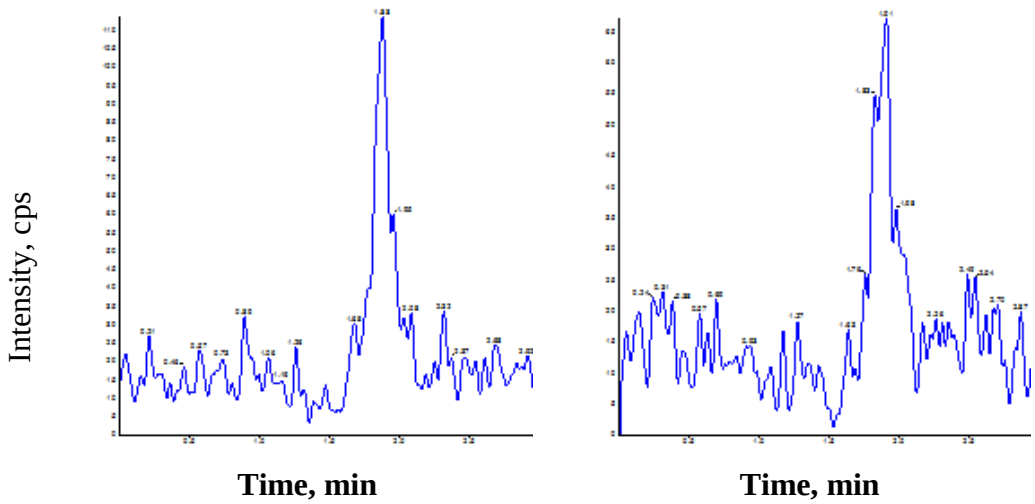
Mass parameters were tuned in both positive and negative ionization modes using electro-spray ionization source. The intense and consistence response was obtained in positive mode since the analyte and internal standard having the ability to accept protons. Protonated form of analyte and the internal standard $[M + H]^+$ ion was the parent ion in the Q_1 spectrum and was used as the

precursor ion to obtain Q_3 product ion spectra. The most sensitive mass transition was observed from m/z 406.1 to 151.1 for nebivolol and from m/z 410.1 to 151.1 for the internal standard. The dwell time for each transition was 200 milli-seconds. The Chromatographic method development includes mobile phase selection, flow rate, column and injection volume. Acetonitrile and methanol were tried in different volume ratio with buffers like ammonium acetate, ammonium formate as well as acid additives like acetic acid and formic acid in varying strengths. It was observed that mobile phase buffer (0.1% formic acid in 5mM ammonium acetate, methanol and acetonitrile (20:20:60, v/v/v). as the mobile phase was most appropriate to give best sensitivity, efficiency and peak shape on Kromasil 100–5C_{18} (100 mm × 4.6 mm, 5 µm) column. The mobile phase was operated at a flow rate of 0.5 mL/min. The retention time for the analyte and the internal standard were low enough (1.86 min) allowing a short run time of 3.0 min. The simple LLE with tert–butyl methyl ether method gave consistent and reproducible recoveries for the analyte and the internal standard from plasma.

Selectivity and chromatography

The selectivity of the method was examined by analyzing blank human plasma extract (Figure. 2A) and an extract spiked only with the IS (Figure. 2B). As shown in Figure. 2A, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug free human plasma at the retention time of the analyte and the internal standard. Similarly, Figure. 2B shows the absence of direct interference from the internals standard to the MRM channel of the analyte. Figure. 2C depicts a representative ion chromatogram for the LLOQ sample (0.051ng/mL).

A



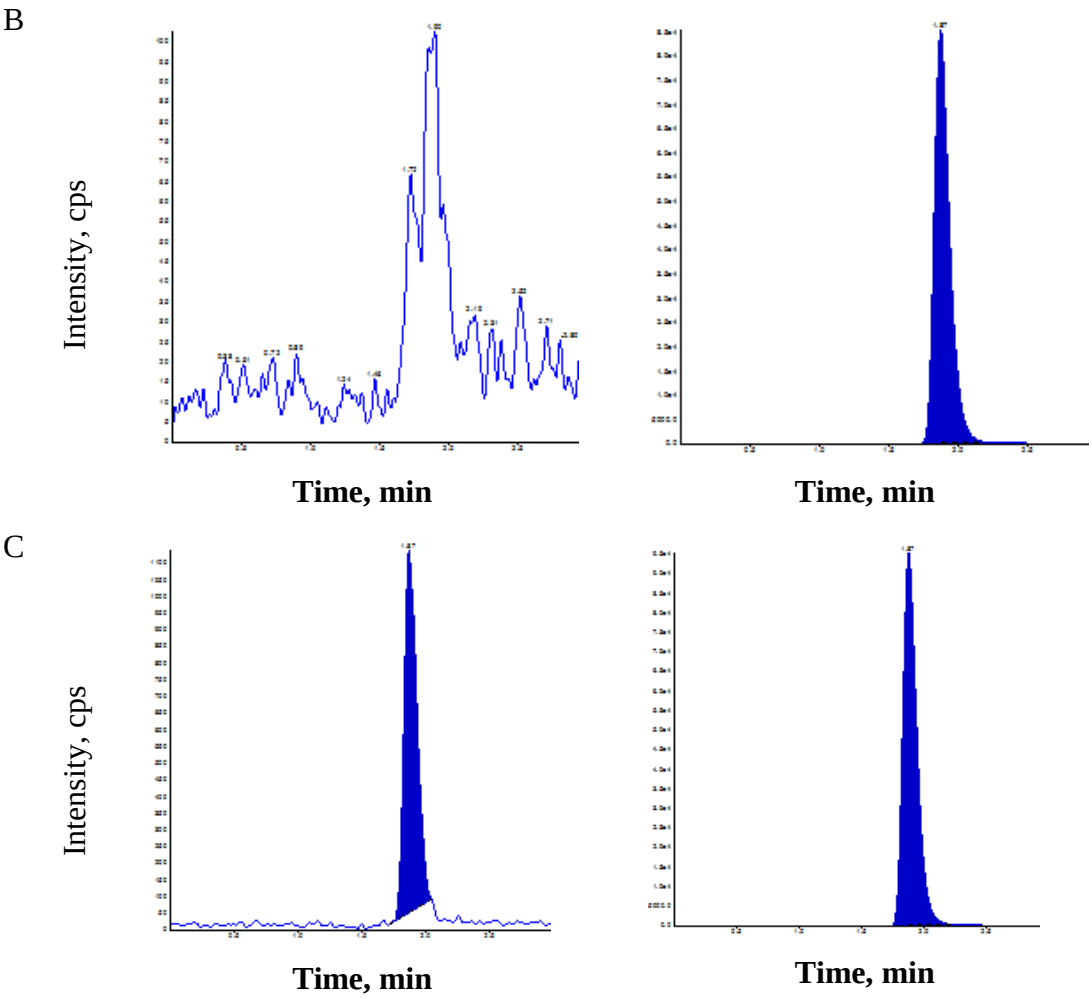


Figure 2: Typical MRM chromatograms of nebivolol (left panel) and IS (right panel) in human blank plasma (A), and human plasma spiked with IS (B), a LLOQ sample along with IS (C).

Matrix effect and sensitivity

Matrix effect assessment was done with the aim to check the effect of different lots of plasma on the back calculated value of QC's nominal concentration. The precision and accuracy for nebivolol at LQC concentration were found to be 2.01% and 101%, and at HQC level they were 1.91% and 101%, respectively. Results revealed that no significant matrix effect was observed in all the six batches of human plasma. The lowest limit of reliable quantification for the analyte was set at the concentration of the LLOQ. The precision and accuracy of analyte at this concentration was found to be 3.78% and 113.73%, respectively.

Linearity, sensitivity, precision and accuracy

The ten point calibration curve was found to be linear over the concentration range of 0.051–10.228 ng/mL for nebivolol. After comparing the two weighting models (1/x and $1/x^2$), a

regression equation with a weighting factor of $1/x^2$ of the drug to the internal standard concentration was found to produce the best fit for the concentration–detector response relationship. The mean correlation coefficient of the weighted calibration curves generated during the validation was ≥ 0.99. Figure 3 shows a representative calibration curve of nebivolol in human plasma. The results for intra-day and inter-day precision and accuracy in plasma quality control samples are summarized in Table 1. The intra-day and inter-day precision deviation values were all within 15% of the relative standard deviation (RSD) at low, middle 1, middle 2 and high quality control level, whereas within 20% at LLOQ QCs level. The intra–day and inter–day accuracy deviation values were all within 100 ± 15% of the actual values at low, middle 1, middle 2 and high quality control level, whereas within 100± 20% at LLOQ QCs level. The results revealed good precision and accuracy.

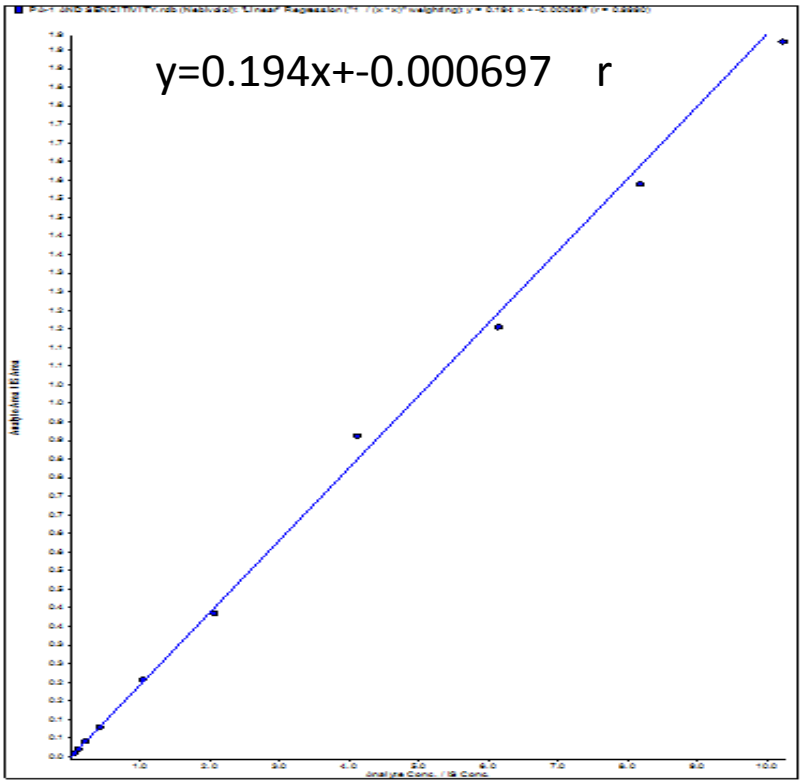


Figure 3: A representative Calibration Curve of nebivolol in human plasma

Table 1: Precession and accuracy of intra and inter day analysis for the determination of nebivolol in human plasma

Analyte	Concentration added(ng/mL)	Intra-day Precision and Accuracy (n=12; 6 from each batch)			Inter-day Precision and Accuracy(n=18; 6 from each batch		
		Conc. found (Mean ± SD; ng/mL)	Precision (%CV)	Accuracy (%)	Conc. found (Mean ± SD; ng/mL)	Precision (%CV)	Accuracy (%)

Nebivolol	0.054	0.0570±0.00186	3.26	105.56	0.0546 ±0.00525	9.62	101.05
	0.135	0.1334±0.00156	1.17	98.83	0.1322 ±0.00438	3.31	97.90
	1.284	1.2557±0.01440	1.15	97.79	1.2598± 0.02049	1.63	98.12
	5.137	5.1111±0.04645	0.91	99.50	5.0920± 0.06791	1.33	99.12
	7.725	7.7740±0.08449	1.09	100.63	7.5907± 0.32826	4.32	98.26

Recovery

Six replicates at low, medium-2 and high quality control concentration for nebivolol was prepared for recovery determination. LLE with *tert*–butyl methyl ether proved to be robust and provided the cleanest samples. The mean overall recovery of nebivolol was 70.93 ± 3.12% with the precision range of 2.1 to 3.06% and the recovery of internal standard was 68.22% with the precision range of 2.42 to 3.72%. The recoveries of analyte and internal standard were good and reproducible.

Stability studies and dilution integrity

In the different stability experiments carried out viz. bench top stability (9 h), auto-sampler stability (51 h), repeated freeze-thaw cycles (4 cycles), reinjection stability (25 h), wet extract stability (46 h at 2–8 °C) and long term stability at –70 °C for 50 days the mean % nominal values of the analyte were found to be within ±15% of the predicted concentrations for the analyte at their LQC and HQC levels (Table 2). Thus, the results were found to be within the acceptable limits during the entire validation. The upper concentration limit of nebivolol can be extended to 16.994 ng/mL by 1/2 and 1/4 dilutions with screened human blank plasma. The mean back-calculated concentrations for 1/2 and 1/4 dilution samples were within 85-115% of their nominal value and the coefficients of variations (%CV) were less than 1%.

Table 2: Stability data for nebivolol in human plasma

Analyte	Stability test	QC (spiked concentration (ng/mL)	Mean ± SD (ng/mL	Precision (%CV)	Accuracy/Stability (%)
Nebivolol	Bench top[a]	0.135	0.1323 ± 0.00163	1.23	98.02
		7.725	7.0925 ± 0.03812	0.54	91.81
	Freeze Thaw[b]	0.135	0.1310 ± 0.00245	1.87	97.04
		7.725	7.0257 ± 0.05641	0.80	90.95
	Wet Extract[c]	0.135	0.1313 ± 0.00216	1.64	97.28
		7.725	7.0488 ± 0.01722	0.24	91.25
	Auto Sampler[d]	0.135	0.1322 ± 0.00183	1.39	97.90
		7.725	7.0590 ± 0.03762	0.53	91.38
	Re injection[e]	0.135	0.1360 ± 0.00179	1.32	100.74
		7.725	7.7900 ± 0.02497	0.32	100.84
	Long term[f]	0.135	0.1318±0.00293	2.22	97.65
		7.725	7.0592±0.04813	0.68	91.44

[a] after 9 h at room temperature; [b] after 4Freeze thaw cycles; [c] after 46 h at 2–8^{0}C ; [d] after 51h at 2–8^{0}C; [e] after 25 h at 2–8^{0}C; at –70^{0}C for 50 days

Stability in whole blood

Stability of nebivolol in whole blood samples was investigated under variety of conditions that could potentially occur in the clinic. 12 mL each of whole blood was spiked with nebivolol at concentration of LQC (0.135 ng/mL) and HQC (7.725 ng/mL). The whole blood samples were then divided into three aliquots (each containing 3.5 mL). Two of these aliquots were then kept at room temperature for 1 h and 3 h and the another aliquot was centrifuged at 1500 rpm, 10 minutes at 4°C and plasma was separated immediately. After 1 h the whole blood samples kept at room temperature was centrifuged at 1500 rpm 10 minutes at 4°C and plasma was separated and the same was repeated for the aliquot which was stored for 3 h.

The six replicates of LQC and HQC level samples of plasma from each was processed as per the extraction procedure described earlier and compare the response ratio of samples kept at room temperature for a period of 1h and 3 h with the fresh (immediately centrifuged) samples. Whole blood samples were stable for 3 h at room temperature (20±5 °C). The percent stability at LQC level was found to be 99.94% with the precision range of 1.67%-1.69% and at HQC level was found to be 99.30% with the precision range of 0.24%-0.68% for 3 h.

CONCLUSION

The LC-MS/MS assay method described in this paper is rapid, simple, specific and reproducible for quantification of nebivolol in human plasma and is validated as per the FDA guidelines. The proposed method described the simple liquid- liquid extraction method with consistent and reproducible recoveries for the analyte from plasma. The method provided good linearity range. The authors can go for more sensitivity LLOQ since, the reported LLOQ is giving intense signal, but the purpose of the proposed method is for bioequivalence and 0.054 ng/mL is quite sufficient for the defined objective. A simple turnaround run time of 3 min makes it an attractive procedure in high throughput bioanalysis of nebivolol.

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