High Response Bio-Analytical Validation approach of Nadolol and Bendroflumethiazide by LC-MS/MS on Rat plasma

Naveen V M K, Veeraswami B*, Srinivasa Rao G
Department of Chemistry, GITAM Institute of Science, GITAM Deemed to be University, Visakhapatnam-45, Andhra Pradesh, India

Article History:
Received on: 23 Aug 2020
Revised on: 23 Oct 2020
Accepted on: 27 Oct 2020

Keywords:
Bendroflumethiazide, Bio-analytical, LC-MS/MS, Nadolol, Rat plasma, Validation

Abstract
A very strong responsive and straightforward the LC-MS / MS assay was developed to and witnessed for that gradation in Nadolol and Bendroflumethiazide in rat plasma. The chromatographic conditions involve isocratic mode using Waters symmetry C18 (150x4.6 mm, 3.5 microns) column. A 0.1 per cent Smartphone process OPA (orthophosphoric Acid) Acetonitrile and in 60:40 is employed, and therefore the detection was administered during +ve mode of electrospray ionisation by using MS. The valid method had validated in the linear range of 8-160 ng/ml Nadolol and 1-20 ng/ml bendroflumethiazide, the precise values considered to be intraday and interday within the acceptable limit. Here these drugs are extracted from the rat plasma by using the liquid-liquid extraction. And these drugs are found stable. Through freeze Thaw, sampler app, vehicle sampler and top of the bench for the future studies. Form of liquid chromatography-tandem mass spectrometry and checked in compliance with guidelines for quantification of food and drug administration Nadolol and Bendroflumethiazide plasma in rats using D6-Nadolol and D6-Bendroflumethiazide as internal standards utilising LC-MS incorporated with quadrupole spectrometer by using electrospray ionisation technique. The target of this analysis is to be done work out the appropriateness of this approach to Nadolol and Bendroflumethiazide Applying Nadolol and bendroflumethiazide and their internal requirements at various quantification stages and retaining different parameters such as instrument durability, precision and accuracy, sample preparation techniques, instrument synchronisation, recovery and matrix effect.

INTRODUCTION
Nadolol was marketed, among others, under the brand Corgard, its structure is shown in Figure 1(A). May be medicine won’t for the prevention of elevated vital sign (Hernandorena et al., 2017; Musini et al., 2019), heart pain and atrial fibrillation (Morin et al., 2016; Chiang et al., 2017). It’s also been used to prevent migraine (Piane et al., 2007) head-aches and complications of cirrhosis (British National Formulary, 2018; Giannelli et al., 2014). It is taken orally. Popular side dizziness has symptoms (James et al., 2017), feeling sluggish, a slow-moving feeling pulse (Ye et al., 2018) and Reynaud syndrome (Wigley and Flavahan, 2016; Linnemann and
Erbe, 2016). Its high intake has serious side effects, coronary Bronchospasm and loss (Haggerty et al., 2003). This is used in breastfeeding, and nursing the defiance is uncertain. Everything is, it's a beta-adrenergic blocker that is non-selective (Wysonge et al., 2017; Bouri et al., 2014) and acts by suppressing adrenergic β1 receptors (Moro et al., 2013) the adrenergic neurons in the heart and β2 in blood vessels.

Nadolol is one among the well-liked β blockers within the control of patients with LQT for QT interval shortening and ventricular arrhythmia avoidance (Batra and Balaji, 2019; Heist and Ruskin, 2010). It’s more operative than cardioselective beta-blockers (Salpeter et al., 2002) such as metoprolol and propranolol within the avoidance of breakthrough Cardiac incidents (Mazzanti et al., 2018). Nadolol has the benefit of daily dosing and thereby increases the weakness of the patient. It is for the person whose function of the kidney decreased, and nadolol could also provide less dose often. For many neurological disorders, such as the avoidance of migraine attacks, attention/deficit/hyperactivity disorder (ADHD), it is effective, and its use has studied as a therapy for tremor and Parkinson’s disease (Sveinbjornsdottir, 2016). Still, neither is well established (Foster et al., 1984).

Bendroflumethiazide formerly, bendrofluazide, brand name Aprinoxmay be a thiamine diuretic won’t treat hypertension, its structure is shown in Figure 1(B). Bendroflumethiazide may be a thiamine diuretic (Dvorak et al., 2007; Moser, 2009) that acts at the start of the distal convoluted tubule (DCT) by inhibiting sodium reabsorption. As a consequence of more sodium hitting the supply ducts, water is lost. Bendroflumethiazide mark has a role to play in the treatment of minor coronary wreck, but the diuretic loop could be safer for overload reduction. The Best use of bendroflumethiazide in hypertension (Sato et al., 2005) at present (part of the result is due to hypertension).

**MATERIALS AND METHODS**

**Natural compounds and reagents**

Acetonitrile and orthophosphoric acid, water (Level HPLC) were purchased from Worli, Mumbai, India, from Merck (India) Ltd. All APIs of Nadolol and Bendroflumethiazide as reference standards were procure from Spectrum pharma research solutions Pvt Ltd, Hyderabad.

**Equipment**

An HPLC system (Waters alliance series e2695) associated Mass Spectrometer with QTRAP the triple quadrupole instrument 5500 (Sciex) was utilised. By the Sciex programming, activity was performed.

**Chromatographic conditions**

Chromatographic separation was administered in aristocratic mode at room temperature, by using an asymmetry C18 column (150x4.6 mm, 3.5 microns). A mix of Acetonitrile and 0.1% OPA in 40: 60 as a mobile process, v / v was used at a flow of 1.0 ml /min. 10 µl was the infused arrangement. The running time was 15 minutes.

**Drafting of the norm and internal control samples**

**Preparation of Bendroflumethiazide parent stock**

10 mg of Bendroflumethiazide standard was weighed and dissolved reliably in 100 ml in a diluent. The Concentration of the solution is 100µg/ml. Take this 0.1ml of the above solution diluted to 10ml with diluent. This is often called the parent stock solution of bendroflumethiazide, and therefore the Concentration is 0.1µg/ml.

**Preparation of Nadolol and Bendroflumethi-azide standard stock solution**

Take 0.4ml Nadolol parent stock solution and 0.4ml of Bendroflumethiazide parent stock solution into 10ml VF and made up to the mark with diluent. It’s a stock solution with Nadolol concentration 320ng/ml and Bendroflumethiazide concentration 40ng/ml. Within the same way, an internal standard stock solution was prepared.

**Preparation of traditional solution**

Normal solutions have been developed by taking 0.5 ml stock solution; 0.5 ml stock solution; 0.5 ml stock solution IS a stock solution, 0.2 ml plasma, 0.3 ml plasma Acetonitrile remaining 0.5 ml diluent in a centrifuged tube and vortexes for 15 min to combine the constituents. After centrifuging 15 min, at 5000 rpm supernatant managed solution was separated and filtered through 0.45 µ nylon syringe filter into a vial and injected into HPLC system.

**Preparation of sample solution**

**Preparation of sample stock**

One tablet (contains 40 mg Nadolol and 5 mg Bendroflumethiazide) was weighed, note the typical weight of the tablet. The tablet was taken into a mortar and crushed into a fine powder. 13.4 mg of tablet powder was weighed accurately and Dissolved in diluents of 100 ml. From this take 0.8 ml and Diluted with diluted at 100 ml. this is often the
sample stock with Nadolol concentration 320 ng/ml and Bendroflumethiazide concentration 40 ng/ml.

**Preparation of sample solution**

For sample preparation takes 0.2 ml plasma, 0.5 ml sample stock, 0.3 ml acetonitrile and 0.5 ml IS, 0.5 ml diluent were taken into a centrifuge tube and vortexed for 15 min to precipitate all the proteins. Centrifuge it for 15 min at 5000 rpm and collect the supernatant solution into a vial and inject it into an HPLC system.

**Method validation**

**Selectivity**

Selectivity was undertaken by investigating the rodent of plasma tests from six-point six, distinct rodents to check for impedance yeah, at the maintenance season in analyses.

**Impact Matrix**

Effect of a matrix for Nadolol and Bendroflumethiazide it was calculated by comparing the height Ratio of Region within plasma samples from six separate drug-free blank plasma samples, and tidy reconstitution samples were collected from the post. At MQC stages, trials were carried out in triplicate with six separate plasma parcels with a satisfactory accuracy of 15 per cent.

**Accuracy and precision**

It was dictated that within monitoring checks (n=6) at a lower quantification limit (LLOQ), low-quality monitoring (LQC), medium quality control (MQC), top quality control (HQC) standards were repeatedly examined. The half CV should be less than 15 per cent and precision should within15 per cent apart from LLQC where it should be within 20 per cent.

**Recovering**

Extracting the productivity in Nadolol and Bendroflumethiazide are determined by an examination of six reproduce at each internal Concentration in power. The share Recovery was measured by matching the height areas of the removed standards with the height areas of the removed standards.

**Carryover**

The analyte held by the chromatographic framework during the infusion of an example that shows up in ensuing clear or obscure examples.

**Dilution integrity**

Dilution integrity ought to be shown by spiking the grid with an analyte focus over the ULOQC and weakening this example with a clear framework.

**Stability**

Stability of stock solution was carried out by looking at Analyses global reaction inside the stability evaluation for the worldwide test reaction arranged through the current product structure. At the LQC and HQC concentration levels, plasma stability tests were carried out using six copies at each dose. If the shift is less than 15 per cent as per US FDA guidance, Analyze was deemed stable. At room temperature, the steadiness of spiked rodent plasma experiments is placed aside; it was calculated for twenty-four hours.

The safety of spiked rat plasma deposited in the autosampler at 2-8°C was measured for twenty-four hours. The durability of the autosampler was tested by looking at the correctly infused extract plasma reports, with the samples re-injected at 2-8°C for twenty-four hours after storage in the autosampler at 2-8°C twenty-four hours. The reproducibility of reinjection was tested by looking at collected plasma tests that were injected promptly, with the samples that were reinvested in the wake of putting away in the autosampler for 24 hours at 2-8°C.

The durability of the cold thaw was led by looking at the steadiness tests that they had been solidified at 30°C except for defrosted multiple occasions, with newly sharp spikes internal samples for monitoring. Six LQC and HQC aliquots per focus the levels have been utilised for the freeze defrost soundness assessment. The Concentration obtained after 24 hours were for long-term stability assessment contrasted and beginning fixation.

**RESULTS AND DISCUSSION**

Lionisation of electrospray having Maximum reaction about reaction air pressure, a mode for chemical ionisation selected in this method. The optimisation of an instrument to offer sensitivity and signal stability during in fusen of analyte within the Continuous-flow to electrospray at all polarities, the ion source worked at the flow 10 μl/min standard chromatogram of Nadolol and Bendroflumethiazide gives more response in positive particle mode in comparison with negative particle model shown in Figure 2.

**Specificity**

Interfering peaks weren’t observed at Nadolol, Bendroflumethiazide, Nadolol-D6, and Bendroflumethiazide-D6 retention times within the chromatogram of blank rat plasma shown in Figure 3. This proved specificity of the tactic to research Nadolol and Bendroflumethiazide simultaneously.
Figure 1: Structure (A) Nadolol and (B) Bendrofluamethiazide Structures

Table 1: Precision and Accuracy of Nadolol

| QC Name       | LLQC (ng/ml) | LQC (ng/ml) | MQC (ng/ml) | HQC (ng/ml) |
|---------------|--------------|-------------|-------------|-------------|
| QC sample-1   | 8.236        | 40.111      | 80.163      | 120.246     |
| QC sample-2   | 8.215        | 40.169      | 80.157      | 120.258     |
| QC sample-3   | 8.225        | 40.118      | 80.146      | 120.247     |
| QC sample-4   | 8.204        | 40.172      | 80.129      | 120.266     |
| QC sample-5   | 8.213        | 40.139      | 80.155      | 120.137     |
| QC sample-6   | 8.247        | 40.158      | 80.194      | 120.174     |
| Mean          | 8.223        | 40.145      | 80.157      | 120.221     |
| SD            | 0.01596      | 0.02605     | 0.02151     | 0.05284     |
| %CV           | 0.19         | 0.06        | 0.03        | 0.04        |
| Accuracy      | 99.84        | 99.93       | 100.25      | 99.89       |

Table 2: Precision and Accuracy of Bendrofluamethiazide

| QC Name       | LLQC (ng/ml) | LQC (ng/ml) | MQC (ng/ml) | HQC (ng/ml) |
|---------------|--------------|-------------|-------------|-------------|
| QC Sample-1   | 1.056        | 5.107       | 10.126      | 15.174      |
| QC Sample-2   | 1.047        | 5.128       | 10.134      | 15.128      |
| QC Sample-3   | 1.029        | 5.139       | 10.117      | 15.139      |
| QC Sample-4   | 1.022        | 5.121       | 10.120      | 15.147      |
| QC Sample-5   | 1.034        | 5.142       | 10.151      | 15.134      |
| QC Sample-6   | 1.018        | 5.127       | 10.148      | 15.124      |
| Mean          | 1.034        | 5.127       | 10.133      | 15.141      |
| Stddev        | 0.01468      | 0.01269     | 0.01431     | 0.01809     |
| %CV           | 1.42         | 0.25        | 0.14        | 0.12        |
| Accuracy      | 99.78        | 100.13      | 100.06      | 100.27      |

Figure 2: Chromatogram of Standard
Figure 3: Chromatogram of Blank
### Table 3: Stability results of nadolol

| Stability experiment spiked plasma | Spiked plasma conc. (n=6, ng/ml) | Conc. measured (n=6, ng/ml) | % CV |
|-----------------------------------|----------------------------------|----------------------------|------|
| **Benchtop stability**            |                                  |                            |      |
| LQC                               | 40                               | 40.126                     | 1.1  |
| MQC                               | 80                               | 80.324                     | 0.47 |
| HQC                               | 120                              | 120.041                    | 0.23 |
| **Autosampler stability**         |                                  |                            |      |
| LQC                               | 40                               | 40.325                     | 0.24 |
| MQC                               | 80                               | 80.187                     | 0.52 |
| HQC                               | 120                              | 120.259                    | 0.29 |
| **Long term (Day 28) stability**  |                                  |                            |      |
| LQC                               | 40                               | 40.375                     | 0.26 |
| MQC                               | 80                               | 80.421                     | 0.14 |
| HQC                               | 120                              | 120.305                    | 0.07 |
| **Wet extract stability**         |                                  |                            |      |
| LQC                               | 40                               | 40.825                     | 0.57 |
| MQC                               | 80                               | 80.327                     | 0.28 |
| HQC                               | 120                              | 120.172                    | 0.17 |
| **Dry extract stability**         |                                  |                            |      |
| LQC                               | 40                               | 40.284                     | 1.55 |
| MQC                               | 80                               | 80.307                     | 0.52 |
| HQC                               | 120                              | 120.478                    | 0.35 |
| **Freeze-thaw stability**         |                                  |                            |      |
| LQC                               | 40                               | 40.296                     | 0.70 |
| MQC                               | 80                               | 80.421                     | 0.36 |
| HQC                               | 120                              | 120.327                    | 0.19 |
| **Short term stability**          |                                  |                            |      |
| LQC                               | 40                               | 40.329                     | 1.11 |
| MQC                               | 80                               | 80.127                     | 0.31 |
| HQC                               | 120                              | 120.478                    | 0.35 |

### Table 4: Stability results of bendroflumethiazide

| Stability experiment spiked plasma | Spiked plasma conc. (n=6, ng/ml) | Conc. measured (n=6, ng/ml) | % CV |
|-----------------------------------|----------------------------------|----------------------------|------|
| **Benchtop stability**            |                                  |                            |      |
| LQC                               | 5                                | 5.017                      | 3.12 |
| MQC                               | 10                               | 10.247                     | 0.86 |
| HQC                               | 15                               | 15.326                     | 0.86 |
| **Autosampler stability**         |                                  |                            |      |
| LQC                               | 5                                | 5.365                      | 3.87 |
| MQC                               | 10                               | 10.249                     | 1.69 |
| HQC                               | 15                               | 15.028                     | 0.99 |
| **Long term (Day 28) stability**  |                                  |                            |      |
| LQC                               | 5                                | 5.268                      | 0.34 |
| MQC                               | 10                               | 10.248                     | 0.37 |
| HQC                               | 15                               | 15.327                     | 0.13 |
| **Wet extract stability**         |                                  |                            |      |
| LQC                               | 5                                | 5.147                      | 2.71 |
| MQC                               | 10                               | 10.469                     | 1.8  |
| HQC                               | 15                               | 15.007                     | 0.74 |
| **Dry extract stability**         |                                  |                            |      |
| LQC                               | 5                                | 5.629                      | 3.07 |
| MQC                               | 10                               | 10.487                     | 2.21 |
| HQC                               | 15                               | 15.326                     | 0.64 |
| **Freeze-thaw stability**         |                                  |                            |      |
| LQC                               | 5                                | 5.471                      | 3.73 |
| MQC                               | 10                               | 10.482                     | 1.55 |
| HQC                               | 15                               | 15.326                     | 0.88 |
| **Short term stability**          |                                  |                            |      |
| LQC                               | 5                                | 5.289                      | 3.24 |
| MQC                               | 10                               | 10.582                     | 0.61 |
| HQC                               | 15                               | 15.327                     | 1.22 |
Table 5: Pharmacokinetic parameters of Nadolol and Bendroflumethiazide

| Pharmacokinetic parameters | Nadolol   | Bendroflumethiazide |
|----------------------------|-----------|---------------------|
| AUC0-t                     | 1216 ng h/ml | 118 ng h/ml        |
| Cmax                       | 60 ng/ml  | 7.7 ng/ml           |
| AUC0-∞                     | 1241 ng h/ml | 122 ng h/ml        |
| T1/2                       | 24 Hr     | 8 Hr                |
| tmax                       | 24 Hr     | 8 Hr                |

In matrix effect, six many rat plasma at LQC and HQC of Nadolol was 99.1, and 99.9 and bendroflumethiazide were 99.8, 99.3%. % CV of both drugs at LQC level were 1.67, 6.93 and HQC level is 0.39, 1.72 respectively. It shows that the matrix effect on the ionisation of the analytes is below the acceptable range, and results are shown in Tables 1 and 2.

Precision and accuracy

The accuracy and precision calculated by pooling all individual assay results of the assay various internal control samples. The accuracy results of nadolol in quality control samples 98.7-99.9 and bendroflumethiazide in quality control samples 99.1-99.9. The half of the CV of Nadolol and Bendroflumethiazide is < 5% altogether internal control samples. Based on the reported data shown in Table 1 for Nadolol & Table 2 for bendroflumethiazide supports the strategy is reliable and precise.

Precision and accuracy

The accuracy and precision calculated by pooling all individual assay results of the assay various internal control samples. The accuracy results of nadolol in quality control samples 98.7-99.9 and bendroflumethiazide in quality control samples 99.1-99.9. The half of the CV of Nadolol and Bendroflumethiazide is < 5% altogether internal control samples. Based on the reported data shown in Table 1 for Nadolol & Table 2 for bendroflumethiazide supports the strategy is reliable and precise.

Ruggedness

The per cent recoveries and per cent CV of Nadolol and Bendroflumethiazide determined with two different analysts and on two different columns were within acceptable criteria in HQC, LQC, MQC and LLQC samples. The per cent recoveries ranged from 99.84-100.72% for nadolol and 99.34%-99.95% for bendroflumethiazide. The %CV values ranged from 0.11-0.38 for nadolol and 0.53-8.23 for bendroflumethiazide. The results proved the method is ruggedness.

Autosampler Carryover

Peak area response of Nadolol and Bendroflumethiazide, Nadolol-D6, Bendroflumethiazide-D6, wasn’t observed within the blank rat plasma samples after successive injections of LLQC and ULQC at the retention times of Nadolol and Bendroflumethiazide, Nadolol-D6, Bendroflumethiazide-D6. Therefore, this method doesn’t exhibit autosampler carryover.

Stability

In solution stability analysis, Nadolol and Bendroflumethiazide solutions were prepared with diluents and put in storage in a refrigerator at 2-8°C. New product options have been correlated with prepared stock solution earlier 24Hrs. The half the shift the nadolol and bendroflumethiazide the stock solutions were steady at 1.26 per cent and 0.79 per cent, respectively up to 24 Hrs when stored in 2-8°C. Benchtop autosampler and stabilities LQC and HQC levels were observed at room temperature Nadolol, and bendroflumethiazide was stable in plasma for twenty-four Hrs in an autosampler at 20°C. From this, it has been verified that at LQC and HQC levels plasma samples correlated with frequent freezing and thawing Nadolol and Bendroflumethiazide their stability has not affected From future stability it had been clear that Nadolol and Bendroflumethiazide were stable up to 24 Hrs at a storage temperature of -30°C. The general stability results of Nadolol and Bendroflumethiazide were tabulated in Tables 3 and 4, respectively.

Pharmacokinetic study

The technique has been approved successfully to measure the Concentration of Nadolol life, as measured by the 0.693 / Kel quotient) and bendroflumethiazide in six many different rats, after oral administration of Nadolol and Bendroflumethiazide sample as an oral dose under fasting condition. After
injecting the drug samples into a rat body collect the samples at different time intervals like 1, 2, 4, 6, 8, 12, 16, 20, 24, 28 and 32 hrs from the rat body. Then according to test technique sample is ready and injected into the chromatographic framework and record the values. The evaluated pharmacokinetic parameters were $C_{max}$ (greatest watched tranquilise focus measured using trapezoidal principle), $t_{max}$ (time to observed most extreme medication focus), $K_{el}$ (evident ϐirst request terminal rate constant determined from the semi-log map of a plasma concentration versus time curves of nadolol shown in Figure 4 and Bendroϐlumethiazide are shown in Figure 5 using the tactic of the smallest amount regression) and $t_{1/2}$ (terminal half The ratio of test/reference for $C_{max}$, $AUC_{0→12}$ and $AUC$ were 88.54 and 94.32 respectively and located to be within the suitable limit of 80%-125%. The further tabulated pharmacokinetic boundaries of Nadolol and Bendroϐlumethiazide are shown in Table 5.

CONCLUSIONS

For the primary time, higher sensitive HPLC-ESI-LCMS/MS method was created and approved for the determination of Nadolol and Bendroϐlumethiazide in rat plasma. Here the described approach is a rugged, fast, reproducible bioanalytical method. A simple and efficient method was developed and may be utilised in pharmacokinetic studies and to see the investigated analyte in body fluids.

ACKNOWLEDGEMENT

The author is thankful to GITAM Deemed to be University for providing necessary facilities to carry out my research work.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

Funding Support

The authors declare that they have no funding sup-
Mazzanti, A., Maragna, R., Vacanti, G., Monteforte, N., Bloise, R., Marino, M., Braghieri, L., Gambelli, P., Memmi, M., Pagan, E., Morini, M., Malovini, A., Ortiz, M., Sacilotto, L., Bellazzi, R., Monserrat, L., Napolitano, C., Bagnardi, V., Priori, S. G. 2018. Interplay Between Genetic Substrate, QTc Duration, and Arrhythmia Risk in Patients With Long QT Syndrome. Journal of the American College of Cardiology, 71(15):1663–1671.

Morin, D. P., Bernard, M. L., Madias, C., Rogers, P. A., Thihalolipavan, S., Estes, I., M, N. 2016. State of the art: atrial fibrillation epidemiology, prevention, and treatment. Mayo Clinic Proceedings, 91:1778–1810.

Moro, C., Tajouri, L., Chess-Williams, R. 2013. Adrenoceptor Function and Expression in Bladder Urothelium and Lamina Propria. Urology, 81(1):211.e1–211.e7.

Moser, M. 2009. Fifty Years of Thiazide Diuretic Therapy for Hypertension. Archives of Internal Medicine, 169(20):1851–1851.

Musini, V. M., Tejani, A. M., Bassett, K., Puil, L., Wright, J. M. 2019. Pharmacotherapy for hypertension in adults 60 years or older: Cochrane Database of Systematic Reviews, (6).

Piane, M., Lulli, P., Farinelli, I., Simeoni, S., Filippis, S. D., Patacchioli, F. R., Martelletti, P. 2007. Genetics of migraine and pharmacogenomics: some considerations. The Journal of Headache and Pain, 8:334–339.

Salpeter, S. R., Ormiston, T. M., Salpeter, E. E. 2002. Cardioselective β-Blockers in Patients with Reactive Airway Disease. Annals of Internal Medicine, 137(9):715–715.

Sato, A., Terata, K., Miura, H., Toyama, K., Lobersica, F. R., Hatoum, O. A., Saito, T., Sakuma, I., Guterman, D. D. 2005. Mechanism of vasodilation to adenosine in coronary arterioles from patients with heart disease. American Journal of Physiology-Heart and Circulatory Physiology, 288(4):H1633–H1640.

Sveinbjornsdottir, S. 2016. The clinical symptoms of Parkinson’s disease. Journal of Neurochemistry, 139:318–324.

Wigley, F. M., Flavahan, N. A. 2016. Raynaud’s Phenomenon. New England Journal of Medicine, 375(6):556–565.

Wiysonge, C. S., Bradley, H. A., Volmink, J., Mayosi, B. M., Opie, L. H. 2017. Beta-blockers for hypertension. Cochrane database of systematic reviews, (1).

Ye, F., Hatahet, M., Youniss, M., Toklu, H., Mazza, J., Yale, S. 2018. The Clinical Significance of Relative Bradycardia. WMJ: Official Publication of the State Medical Society of Wisconsin, 117:73–78.