

RESEARCH ARTICLE

Open Access



An approach to select linear regression model in bioanalytical method validation

Sandeep S. Sonawane*, Santosh S. Chhajed, Sana S. Attar and Sanjay J. Kshirsagar

Abstract

Background: The accuracy of any bioanalytical method depends on the selection of an appropriate calibration model. The most commonly used calibration model is the unweighted linear regression, where the response (y -axis) is plotted against the corresponding concentration (x -axis). The degree of association between these two variables is expressed in terms of correlation coefficient (r^2). However, the satisfactory r^2 alone is not adequate to accept the calibration model. The wide calibration curve range used in the bioanalytical methods is susceptible to the heteroscedasticity of the calibration curve data. The use of weighted linear regression with an appropriate weighting factor reduces the heteroscedasticity and improves the accuracy over the selected concentration range.

Methods: The present work describes a rapid and simple RP-HPLC method for the estimation of chlorthalidone in spiked human plasma. The calibration curve standards were studied in the concentration range of 100–3200 ng/mL. The chromatography was performed on a C18 column (250 × 4.6 mm, 5 μm) in an isocratic mode at a flow rate of 1 mL/min using methanol:water (60:40%, v/v) as a mobile phase. The detection was carried out at 276 nm. Both the unweighted regression model and weighted regression models with different weighting factors ($1/x$, $1/\sqrt{x}$, and $1/x^2$) were evaluated for heteroscedasticity. The statistical approach for the selection of a suitable regression model with appropriate weighting factors was discussed and the developed bioanalytical method was further validated, as per US-FDA guidelines.

Results: In calibration curve experiments, although the acceptable r^2 of 0.998 was obtained, the % residual plot showed that the data were susceptible to heteroscedasticity. When the weighted linear regression was applied to the same calibration curve data set, no significant difference between % relative residual (% RR) was observed. Furthermore, when % relative error (% RE) was calculated for different weighting factors, it was observed that the regression model with $1/x$ weighting factor gave a minimum % RE. The calibration curve was found to be linear in the range of 100 to 3200 ng/mL. The validation experiments proved good accuracy, and intra- and inter-day variability and acceptable recovery. Stability studies proved that the drug was stable under tested stability cycles.

Conclusions: From the statistical reports obtained from the present work, it was observed that the calibration curve in bioanalytical experiments was susceptible to heteroscedasticity using the unweighted linear regression model. Hence, to obtain homoscedasticity in the calibration curve experiments, there is a need for a weighted linear regression model. The appropriate regression model was further selected by evaluating the % RE for different weighting factors.

Keywords: Bioanalytical method validation, Linear regression model, Homoscedasticity, Weighting factor, HPLC, Chlorthalidone

* Correspondence: sandeeps.iop@gmail.com

MET's Institute of Pharmacy, MET League of Colleges, Bhujbal Knowledge City, Adgaon, Nashik, Maharashtra 422 003, India

Introduction

While performing any bioanalytical method, the concentration of drug present in a biological matrix is determined by extracting the drug, either by solid-phase extraction (SPE), liquid-liquid extraction (LLE), or protein precipitation (PP). The selection of calibration model in bioanalysis should be done as part of the method development prior to the method validation (Nagaraja et al. 1999). The response obtained (peak area ratio or peak height ratio) (y -value: dependent variable) is plotted against the corresponding concentration (x -value: independent variable). The relationship is established as $y = bx + a$ for the obtained calibration curve, where b is the slope and a is the intercept. This simple method is called the method of least squares and is the most commonly used regression model (Kimanani 1998).

The concentrations used to generate a calibration curve are the calibration curve standards with known concentrations. The degree of association between y and x variables is expressed in terms of correlation coefficient (r^2) and is considered acceptable for an r^2 greater than 0.99. The unknown samples are typically evaluated using the regression results obtained from the calibration curve. However, the correlation coefficient value is not always sufficient to accept the linear regression model. Usually, in bioanalytical experiments, when drug concentrations are evaluated in a wide concentration range, the heteroscedasticity of the data is expected, where the variance increases with rising concentrations. Therefore, despite the acceptable correlation coefficient, the use of unweighted linear regression for the calibration curve experiment with heteroscedasticity will generate inaccurate analytical results, especially at a lower concentration range (Boulanger et al. 2003).

To handle this situation, two approaches were used; the use of weighted linear regression and the use of polynomial equations (Nakov et al. 2014, Korany et al. 2013). The latter is only applied in the case of non-linear or curved data sets, where the quadratic regression $y = a + bx + cx^2$ is the most preferable. However, higher order polynomial equations greater than quadratic are generally not preferred (Singtoroj et al. 2006). The data set generated in the bioanalytical method is linear and,

Table 1 % Recovery of chlorthalidone and internal standard in different LLE solvents

| Sr. No. | LLE solvent | % Recovery of chlorthalidone | % Recovery of internal standard |
|---------|--------------------------------|------------------------------|---------------------------------|
| 1 | Ethyl acetate | 67 | 59 |
| 2 | <i>ter</i> -butyl methyl ether | 23 | 19 |
| 3 | Diethyl ether | 16 | 7 |
| 4 | Chloroform | 8 | 6 |
| 5 | Tetrahydrofuran | 5 | 3.5 |

Table 2 Area ratios from calibration experiments

| CC No. | Conc. (ng/mL) | Area ratios ($n = 6$) | % RSD |
|--------|---------------|-------------------------|-------|
| 1 | 100 | 0.0555 | 10.59 |
| 2 | 200 | 0.1048 | 6.79 |
| 3 | 400 | 0.1921 | 13.80 |
| 4 | 800 | 0.3470 | 4.30 |
| 5 | 1600 | 0.7498 | 4.67 |
| 6 | 3200 | 1.5293 | 5.62 |

therefore, the most commonly used method is the weighted linear regression. The choice of weighting factor in the weighted linear regression evaluated in the pre-validation stage is based on evaluating the % relative error (% RE) and the calibration model on the test of homoscedasticity (Almeida et al. 2002).

The % RE is calculated for each calibration standard as follows:

$$\%RE = \frac{C(\text{found}) - C(\text{nom})}{C(\text{nom})} \times 100$$

where $C(\text{found})$ is the back-calculated concentration and $C(\text{nom})$ is the nominal concentration of the calibration standard. The most suitable weighting factor is the one that gives the minimum total % RE.

The test of homoscedasticity was performed by plotting % relative residual (% RR) vs. concentration and by applying the F test as follows:

$$F(\text{exp}) = \frac{s_2^2}{s_1^2}$$

where $F(\text{exp})$ is the experimental F value expressed as a ratio of variance obtained at the lowest concentration level (s_1^2) and at the highest concentration level (s_2^2) of the working range. The $F(\text{exp})$ is compared with the $F(\text{tab})$, which is obtained from the F -table at a confidence level of 99% for $f_1 = f_2 = (n - 1)$ degrees of freedom. The test of homoscedasticity is accepted for $F(\text{exp}) < F(\text{tab})$. In this case, the variance is constant over the entire calibration range and the residual will fall randomly around the x -axis.

The objective of this work was to discuss a statistical approach for the selection of linear regression model during bioanalytical method development. The proposed approach was based on the study of heteroscedasticity for the calibration data by using the unweighted regression model and to suggest the need for a weighted regression model to reduce heteroscedasticity. The proposed work also elaborates the selection of weighting factor in weighted linear regression, the choice of optimum regression model using total % RE, and a test of homoscedasticity for the given calibration data set. Chlorthalidone was used as a model drug in this study.

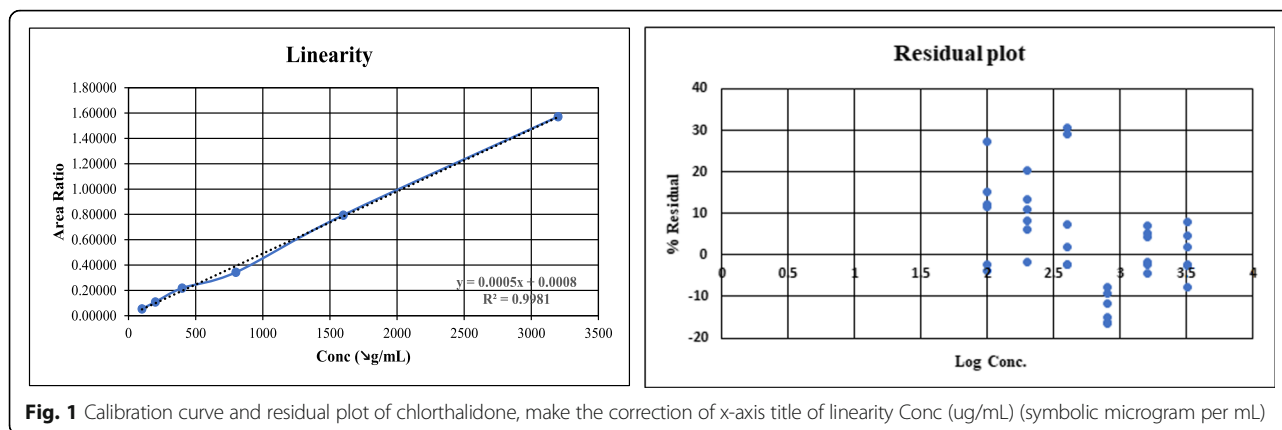


Fig. 1 Calibration curve and residual plot of chlorthalidone, make the correction of x-axis title of linearity Conc (µg/mL) (symbolic microgram per mL)

The drug was extracted from spiked human plasma using a liquid-liquid extraction technique and estimated using the RP-HPLC method.

Chemically, chlorthalidone is 2-chloro-5-(2,3-dihydro-1-hydroxy-3-oxo-1H-isoindol-1-yl) benzene sulfonamide (O'Neil et al. 2006), a diuretic agent used in the treatment of hypertension and renal disorders. Several analytical methods have been described for the estimation of chlorthalidone in biological fluids (Dadgar and Kelly 1988) and chlorthalidone in combination with other antihypertensive agents (Giachetti et al. 1997; Khuroo et al. 2008; El-Gindy et al. 2008; Gonzalez et al. 2010; Elgawish et al. 2011; Mhaske et al. 2012; Belal et al. 2012; Ramakrishna et al. 2015; Shah et al. 2016; Patel et al. 2017; Aruna et al. 2017).

Experimental

Materials and methods

The working standards of chlorthalidone and guaifenesin (used as an internal measure) were obtained as a gift sample from Glenmark Pharmaceuticals Ltd., Nashik. Human plasma samples were procured as a gift sample from NDMVP's Dr. Vasantrao Pawar Medical College, Hospital and Research Centre, Nashik and pooled plasma was prepared by thoroughly mixing plasma from six different sources. HPLC grade methanol was purchased from s d fine-chem Ltd., Mumbai. Double-distilled water used was prepared freshly using the All Glass Double Distillation Assembly, purchased from Borosil India Pvt. Ltd., Mumbai and filtered through a Durapore membrane filter

(0.45 µm × 47 mm), purchased from Axiva Scichem Biotech, New Delhi.

The HPLC system consisted of two pumps PU-2080 plus (JASCO Corporation, Japan) fitted with a 100 µL Rheodyne Loop injector (7725i) and LC-Net II/ADC system control. Detection was carried out on a UV-2075 detector (JASCO Corporation, Japan) and the data acquisition was performed using BORWIN chromatography software (Version 1.50).

All chromatographic separations were carried out on a Phenomenex Kinetex C18 column (250 × 4.6 mm, 5 µm) using a mobile phase consisting of methanol:water (60:40%, v/v) in an isocratic mode at a flow rate of 1 mL/min. All eluents were detected at 276 nm.

Preparation of standard stock solution and working standard solutions

The standard stock solution of 1000 µg/mL of chlorthalidone and guaifenesin (internal standard) was prepared by dissolving 10 mg of chlorthalidone and guaifenesin in separate 10 mL volumetric flasks using methanol.

Furthermore, the above-prepared standard stock solution of chlorthalidone was appropriately diluted with a methanol to get working standard solutions of concentrations of 1, 2, 4, 8, 16, and 32 µg/mL. Likewise, the standard stock solution of the internal standard was appropriately diluted with methanol to obtain a working standard solution of 20 µg/mL.

Table 3 Regression parameters for unweighted and weighted regression with different weighting factors

| Sr. No. | Weighting factor | <i>a</i> | <i>b</i> | <i>r</i> ² | % RE | <i>F</i> (exp) |
|---------|--------------------------|----------|----------|-----------------------|--------|----------------|
| 1 | 1 | -0.00083 | 0.00048 | 0.998 | 294.95 | 272.95 |
| 2 | 1/ <i>x</i> | +0.00415 | 0.00049 | 0.999 | 98.70 | 0.26 |
| 3 | 1/ <i>x</i> ² | +0.00872 | 0.00046 | 0.998 | 101.87 | 0.00026 |
| 4 | 1/√ <i>x</i> | -0.00074 | 0.00048 | 0.997 | 169.51 | 8.52 |

a is the intercept and *b* is the slope of calibration curve

Table 4 Blank responses and LLOQ peak areas for chlorthalidone

| Sr. No. | Blank response ($\mu\text{V s}$) | Peak areas at LLOQ ($\mu\text{V s}$) |
|---------|------------------------------------|--|
| 1 | 11,471 | 251,801 |
| 2 | 27,247 | 349,581 |
| 3 | 22,174 | 286,879 |
| 4 | 12,742 | 319,938 |
| 5 | 17,960 | 332,526 |
| 6 | 25,939 | 302,156 |

Sample preparation and data analysis

Aliquots of 1 mL of pooled blank plasma were taken in stoppered glass tubes of 20 mL capacity. To these tubes, 125 μL of working standard solutions was added appropriately to get a six-point calibration curve (CC) of standards of chlorthalidone with concentrations of 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 $\mu\text{g}/\text{mL}$, respectively. In each calibration curve standard solution, 125 μL of 20 $\mu\text{g}/\text{mL}$ working standard solution of internal standard was added and vortex-mixed for 5 min.

Similarly, the quality control (QC) samples were prepared, which consist of a lower limit of quantitation (LLOQ), lower quality control concentration (LQC), middle quality control concentration (MQC), and higher quality control sample (HQC). All QC samples were analyzed in five replicates for each QC level.

Liquid-liquid extraction experiments

An aliquot of 1 mL of blank human plasma was removed in a 20 mL stoppered glass tube and spiked with 125 μL of 100 $\mu\text{g}/\text{mL}$ methanolic solution of chlorthalidone and 125 μL of 20 $\mu\text{g}/\text{mL}$ methanolic solution internal standard. This mixture was vortex-mixed for 5 min. Furthermore, 5 mL aliquots of liquid-liquid extraction (LLE) solvent were added to each stoppered

tube which were vortex-mixed for 5 min; then, the tubes were centrifuged at 3000 rpm for 10 min at 4 $^{\circ}\text{C}$ in a cooling centrifuge to achieve phase separation. From this solution, 2 mL of the organic layer was transferred to another tube and the organic solvent was evaporated under the nitrogen stream.

The residue obtained upon evaporation to dryness was reconstituted with 500 μL of the mobile phase and 100 μL was injected into the chromatographic system under optimal chromatographic conditions.

Calibration curve and selection of calibration model

In calibration runs, all CC standards were analyzed in six replicates using an optimized LLE experiment and chromatographic conditions.

At the end of calibration runs, the chromatograms of CC standards were processed to obtain the peak areas for chlorthalidone and the internal standard. For each CC standard, the area ratio of chlorthalidone to internal standard was calculated and the six-point calibration curve was constructed from the peak area ratio vs. respective concentration of CC standard.

The CC standard data were studied using unweighted linear regression and weighted linear regression with a weighting factor of $1/x$, $1/\sqrt{x}$, and $1/x^2$. The data analysis was carried out using Microsoft $^{\circ}$ Excel $^{\circ}$ 2016 MSO (64-bit).

Validation

The developed method was validated as per the Guidance for Industry: Bioanalytical Method Validation (FDA and Food and Drug Administration, May 2018). Selectivity was studied at the lower limit of quantification (LLOQ) of 100 ng/mL by comparing blank responses of plasma from six different sources with peak areas

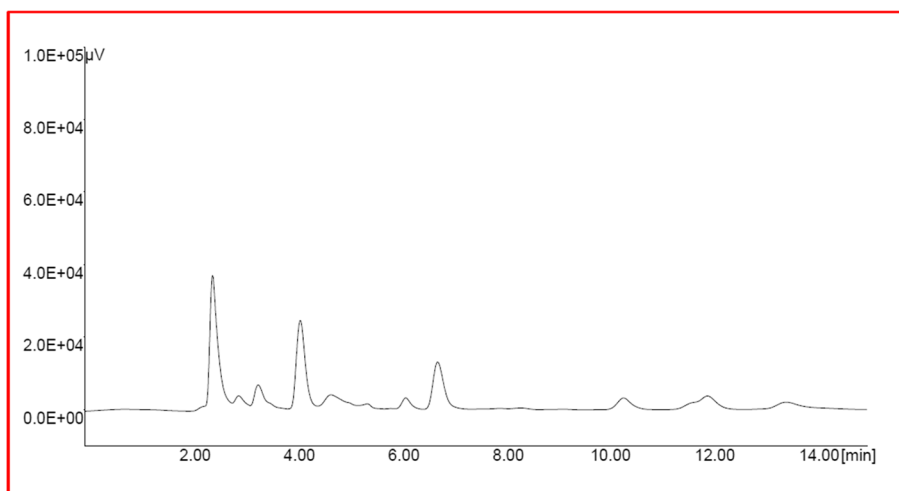
**Fig. 2** Representative chromatogram of blank plasma extract

Table 6 Stability studies for chlorthalidone ($n = 5$)

| Level | Stability at room temperature | | | | | | Stability at $-20\text{ }^{\circ}\text{C}$ | | | | | | Freeze thaw (FT) stability | | | | | |
|-------|-------------------------------|--------|-------|-------|------|-------|--|---------|---------|---------|---------|---------|----------------------------|-------|--------|-------|------|------|
| | % Nominal | | | % RSD | | | % Nominal | | | % RSD | | | % Nominal | | | % RSD | | |
| | 2 h | 4 h | 6 h | 2 h | 4 h | 6 h | 10 days | 20 days | 30 days | 10 days | 20 days | 30 days | FT1 | FT2 | FT3 | FT1 | FT2 | FT3 |
| LQC | 98.84 | 98.105 | 98.38 | 2.31 | 1.74 | 1.546 | 96.74 | 96.968 | 98.91 | 1.03 | 3.51 | 1.65 | 90.910 | 95.06 | 98.81 | 1.18 | 1.54 | 1.98 |
| HQC | 100.8 | 100.6 | 100.6 | 2.48 | 0.48 | 1.678 | 100.50 | 100.31 | 100.7 | 0.36 | 2.81 | 0.23 | 100.49 | 100.4 | 100.45 | 0.17 | 0.87 | 0.28 |

LLOQ: 400 ng/mL), MQC (50% of the CC range: 1600 ng/mL), and HQC (near to the upper boundary of the calibration curve: 3200 ng/mL).

Optimization of liquid-liquid extraction experiments

The LLE solvents investigated were ethyl acetate, *ter*-butyl methyl ether, diethyl ether, chloroform, and tetrahydrofuran. Good recovery was obtained in ethyl acetate and thus selected for use as an extraction solvent in LLE. The % recovery for chlorthalidone and the internal standard in different organic solvents is shown in Table 1.

Calibration curve study and selection of optimal regression model

When the data obtained from the CC standards (Table 2) were subjected to unweighted linear regression, it was found that the % RR for the lowest concentration was 31.20%, compared with 0.18% of the highest concentration (Fig. 1). The % RR was calculated as:

$$\%RR = \frac{Y(\text{found}) - Y(\text{nom})}{Y(\text{nom})} \times 100$$

This proved that inaccurate results may be generated when unweighted linear regression is used, even with an acceptable r^2 . Also, when the CC standard data of unweighted regression were subjected to the test of homoscedasticity, the $F(\text{exp})$ value found 272.95 which was significantly higher than $F(\text{tab}) = 5.05$ ($F_{5, 5}$). This revealed that the variance was not evenly distributed over the CC standard range and resulted in heteroscedasticity. Therefore, from the % RR and from the test of homoscedasticity, it can be reasoned that there is a need to apply a weighted regression model for the given calibration data set.

The weighted regression models with a weighting factor of $1/x$, $1/\sqrt{x}$, and $1/x^2$ were further constructed on the same calibration data set.

Furthermore, for the selection of the optimal calibration model, the weighted linear calibration model with the minimum % RE and $F(\text{exp})$ was selected. The regression parameters for unweighted and weighted regressions with % RE and F value are presented in Table 3.

It was noted that the weighted regression model with a weighting factor of $1/x$ has the lowest % RE and is suitable to homogenize the variance of the residual. Thus, it was decided to adopt a calibration model of weighted linear regression with a weighting factor of $1/x$ in the calibration range of 100 ng/mL to 3200 ng/mL.

Validation

From Table 4, it was observed that the peak areas for LLOQ samples were five times higher than blank responses from the plasma. Thus, it was concluded that the method was selective at an LLOQ of 100 ng/mL.

The chromatogram of blank plasma extract is shown in Fig. 2, which revealed the lack of significant interference at the retention times of chlorthalidone and the internal standard. The representative chromatogram of MQC sample is presented in Fig. 3.

The results of accuracy, precision, and the extraction recovery are shown in Table 5. It was found that the intra-day and inter-day % RE was between $\pm 15\%$ and the % RSD was less than 15%. Hence, this proved the acceptable recovery of chlorthalidone and of the internal standard.

The results of stability studies are shown in Table 6, which showed that the % nominal concentration was between 90 and 98.84% with a % RSD less than 15%. This concluded that the drug remained stable after stability cycles.

Conclusions

In the present work a simple, rapid and accurate RP-HPLC method was described for the estimation of chlorthalidone in spiked human plasma using liquid-liquid extraction. Also, it was demonstrated that in the calibration curve experiments using bioanalytical methods, the calibration data suffered from heteroscedasticity due to the broad concentration range. The heteroscedasticity can be minimized by using the weighted linear regression model. The weighted regression model with appropriate weighting factors can be selected by studying the % RE and testing for homoscedasticity. The developed method performed well with selectivity, precision, accuracy, and linearity in the chlorthalidone concentration range from 100 to 3200 ng/mL and the heteroscedasticity was minimized by using a weighted regression model with a weighting factor of $1/x$.

Abbreviations

% RE: % Relative error; % RR: % Relative residual; % RSD: % Relative standard deviation; CC: Calibration curve; HQC: Higher quality control concentration; LLE: Liquid-liquid; LLOQ: Lower limit of quantitation; LQC: Lower quality control concentration; MQC: Middle quality control concentration; PP: Protein precipitation; QC: Quality control; SPE: Solid-phase extraction

Acknowledgements

Authors are thankful to the Management and Trustees of Mumbai Educational Trust's Bhujbal Knowledge City, Nashik, for providing necessary chemicals and analytical facilities, Glenmark Pharmaceuticals Ltd., Nashik for providing gift samples of drug and internal standard, to NDMVP's Dr. Vasantrao Pawar Medical College, Hospital and Research Centre, Nashik, for providing human plasma samples.

Funding

The research work mentioned in this paper is not funded by any funding agency and is financed by institute.

Availability of data and materials

Please contact author for data requests.

Authors' contributions

SS designed the experiments, performed data analysis, and drafted the manuscript. SA performed the experiments and assisted with data analysis. SCS performed the literature survey and assisted in designing the protocol of experiments. SK provided the guidance in designing and drafting the manuscript. All authors of this work read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 4 September 2018 Accepted: 26 November 2018

Published online: 03 January 2019

References

- Almeida AM, Castel-Branco MM, Falcão AC. Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2002;774:215–22.
- Aruna G, Bharathi K, Prasad K. Development and validation of bioanalytical method for simultaneous estimation of cilnidipine and nebivolol in human plasma. *Int J Pharm Pharm Sci.* 2017;9(10):253–9.
- Belal F, El-Brashy AM, El-Enany N, Tolba MM. Simultaneous determination of celioprolol HCl and chlorthalidone in tablets and biological fluids using high-performance liquid chromatography. *Acta Chromatograph.* 2012; 24(2):185–206.
- Boulangier B, Chiap P, Dewe W, Crommen J, Hubert P. An analysis of the SFSTP guide on validation of chromatographic bioanalytical methods: progresses and limitations. *J Pharm Biomed Anal.* 2003;32:753–65.
- Dadgar D, Kelly MT. Determination of chlorthalidone in human plasma by reversed-phase micellar liquid chromatography. *Analyst.* 1988;113(8):1223–7.
- Elgawish MS, Mostafa SM, Elshanawane AA. Simple and rapid HPLC method for simultaneous determination of atenolol and chlorthalidone in spiked human plasma. *Saudi Pharm J.* 2011;19:43–9.
- El-Gindy A, Sallam S, Abdel-Salam RA. HPLC method for the simultaneous determination of atenolol and chlorthalidone in human breast milk. *J Sep Sci.* 2008;31:677–82.
- Food and Drug Administration (2018) Bioanalytical method validation: guidance for industry, U.S. Department of Health and Human Services <https://www.fda.gov/downloads/drugs/guidances/ucm070107.Pdf>. Accessed 15 June 2018.
- Giachetti C, Tenconi A, Canali S, Zanolo G. Simultaneous determination of atenolol and chlorthalidone in plasma by high-performance liquid chromatography application to pharmacokinetic studies in man. *J Chromatogr B Biomed Sci Appl.* 1997;698:187–94.
- Gonzalez O, Iriarte G, Rico E, Ferreirós N, Maguregui MI, Alonso RM, Jiménez RM. LC–MS/MS method for the determination of several drugs used in combined

- cardiovascular therapy in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2010;878:2685–92.
- Hygriton Chlorthalidone 25 mg tablets. (n.d.) <http://www.medsafe.govt.nz/profs/Datasheet/h/Hygrotontab.pdf>. Accessed 31 Mar 2018.
- Khuroo A, Mishra S, Singh O, Saxena S, Monif T. Simultaneous determination of atenolol and chlorthalidone by LC–MS–MS in human plasma. *Chromatographia.* 2008;68:721.
- Kimanani EK. Bioanalytical calibration curves: proposal for statistical criteria. *J Pharm Biomed Anal.* 1998;16:1117–24.
- Korany MA, Maher HM, Galal SM, Ragab MA. Comparative study of some robust statistical methods: weighted, parametric, and nonparametric linear regression of HPLC convoluted peak responses using internal standard method in drug bioavailability studies. *Anal Bioanal Chem.* 2013;405(14): 4835–48.
- Mhaske RA, Garole DJ, Mhaske AA, Sahasrabudhe S. RP-HPLC method for simultaneous determination of amlodipine besylate, valsartan, telmisartan, hydrochlorothiazide and chlorthalidone: application to commercially available drug products. *Int J Pharmaceut Sci.* 2012;3(1):141–9.
- Nagaraja NV, Paliwal JK, Gupta RC. Choosing the calibration model in assay validation. *J Pharm Biomed Anal.* 1999;20:433–8.
- Nakov N, Tonic-Ribarska J, Dimitrovska A, Petkovska R. Statistical approach for selection of regression model during validation of bioanalytical method. *Maced. pharm. bull.* 2014;60(1):19–25.
- O'Neil JM, Heckelmen PF, Koch CB, Roman KJ, Kenny CM, D'Arecca MR. The Merck index, an encyclopedia of chemicals, drugs and biologicals, 14th ed. Merck & co, Inc. NJ, USA: Whitehouse station; 2006. p. 2193.
- Patel B, Jangid AG, Suhagia BN, Desai N. The simultaneous UPLC–MS/MS determination of emerging drug combination; candesartan and chlorthalidone in human plasma and its application. *Biomed Chromatogr.* 2017;31(9):e3946.
- Ramakrishna R, Kumar Puttrevu S, Bhaterra M, Bala V, Sharma VL, Bhatta RS. Simultaneous determination of azilsartan and chlorthalidone in rat and human plasma by liquid chromatography–electrospray tandem mass spectrometry. *J Chromatogr B.* 2015;990:185–97.
- Shah JV, Patel DP, Shah PA, Sanyal M, Shrivastav PS. Simultaneous quantification of atenolol and chlorthalidone in human plasma by ultra-performance liquid chromatography–tandem mass spectrometry. *Biomed Chromatogr.* 2016; 30(2):208–16.
- Singtoroj T, Tarning J, Annerberg A, Ashton M, Bergqvist Y, White NJ, Lindegardh N, Day NP. A new approach to evaluate regression models during validation of bioanalytical assays. *J Pharm Biomed Anal.* 2006;11:219–27.

Submit your manuscript to a SpringerOpen® journal and benefit from:

- Convenient online submission
- Rigorous peer review
- Open access: articles freely available online
- High visibility within the field
- Retaining the copyright to your article

Submit your next manuscript at ► springeropen.com