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Development and Validation of a Bioanalytical Method for the Determination of Nimodipine Concentration in Plasma of Rats

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Abstract

Nimodipine is a calcium channel blocker used for the prevention of cerebral vasospasm after subarachnoid hemorrhage, a type of strokes. Its unique ability to selectively dilate blood vessels in the brain makes it a crucial medication in neurology. Pharmaceutical analysis of nimodipine is important to ensure its safety, efficacy, and quality, and to establish optimal dosage regimens for patients. Hence, a bioanalytical method based on high performance liquid chromatography (HPLC) was developed and validated to determine the concentration of nimodipine in rat's plasma that has been spiked with the drug. The current method is distinguished from the previously published methods in two issues. First, sample preparation involved the utilization of 1-pentanol as an extraction solvent for the processing of plasma. Second, nifedipine was used as an internal standard (IS) during chromatographic separation on octadecylsilane (ODS) column and water/methanol solution as the mobile phase. The validation results indicated that the proposed method is specific and linear for nimodipine within the concentration range of 10.0 to 150.0 ng/mL. The accuracy and precision of the method were assessed for both inter-day and intra-day measurements. The results revealed accuracies ranging from 97.00% to 99.66% and 95.12% to 101.28%, respectively. In terms of precision, the values ranged from 1.17% to 3.10% for inter-day measurement and 1.00% to 2.23%, for intra-day measurements. The recovery rates for nimodipine and nifedipine were 95.91% and 86.61%, respectively. Furthermore, the method demonstrated good stability at short-term room temperature (99.44%), long-term freeze temperature (97.80%), and freeze/thaw (97.04%) conditions. Overall, these findings suggest that the method is suitable for use in pre-clinical pharmacokinetic studies involving nimodipine-containing drug delivery system.

Keywords: Bioanalytical method, HPLC, Nimodipine, pre-clinical pharmacokinetics, validation.

Introduction

In pharmacokinetics, bioavailability or bioequivalence studies, the concentration of drug is necessary to be determined in the biological fluids through suitable bioanalytical methods. Therefore, such methods must be validated to meet its objectives [(1)]. The validation is essential since plasma or other biological samples vary in their composition; thus method validation ensure reliability and repeatability of the analysis [(2)]. The plasma of rats is utilized since preliminary studies are widely performed in this such animal [(3)] and the knowledge of pharmacokinetics is essential for obtaining safe and effective drug products [(4)] . Sample processing is the first step for bioanalytical method development, which improve the sensitivity of the method through the enhancement of analyte's concentration, as well as to get rid of interferences [(5)].

This is of prime importance when the concentration of drug is very low [(6)]. Therefore, in the next chromatography step, the signal can be differentiated from background noise. Precipitation of proteins and liquid-liquid extraction are commonly employed simple techniques and do not require sophisticated systems [(7)]. A solvent with justified properties solvent A On the other hand, the use of solid phase extraction (SPE) cartridges is lengthy and adds more cost to the whole procedure [(8)]. Drugs with defined chromophores absorb radiation in the near ultraviolet (UV) region and thus are suitable for detection after liquid chromatography of the processed biological samples such as plasma [(9)]. Therefore, UV detectors are the most commonly used detectors in HPLC systems since most drug molecules absorb UV radiation [(10)]. Although other detectors such as mass spectrometry (MS) detectors are characterized by higher resolution, sensitivity and selectivity, but are much more expensive, difficult to maintain, need extensive training to operate, and have high operation costs [(11)]. Subarachnoid hemorrhage is a type of cerebrovascular disorder which is detected

and evaluated with computed tomograpic angiography [12]. Nimodipine is chemically 1,4-dihydropyridine dug, synthesized by H. Meyer et al [13] approved for the prevention of cerebral vasospasm following subarachnoid hemorrhage [14]. As calcium channel antagonist, studies showed that the drug reduces the risk of poor outcome and delayed cerebral ischemia [15]. The higher partition coefficient of nimodipine compared to that of nifedipine (Figure 1) stands for its ability to reach the central nervous system [16].

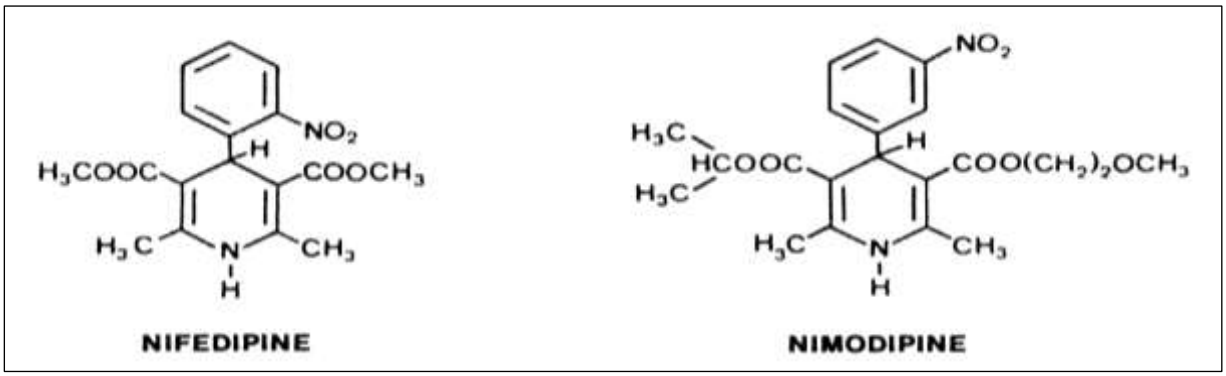

Figure 1. Chemical structures of nimodipine and nifedipine.

The log P (octanol/water) value of nimodipine is 3.05 and the drug is soluble in ethyl acetate, sparingly soluble in absolute alcohol, but insoluble in water [17]. Apart from marketed oral and injectable products, the drug was investigated in solid dispersion, solid lipid nanoparticles, nanoemulsion, nanoliposomes, and nanocrystals in order to improve the solubility of the drug [18]. An array of organic solvents was studied for the extraction of 1,4-dihydropyridine calcium antagonists through factorial design [19]. However, 1-pentanol or other alcohols were not studied. In addition, the previous HPLC methods which had been reported for the determination of nimodipine concentration in plasma utilized sophisticated techniques [20]; need long time, or employ mobile phases with adjusted buffer solutions [21]. To avoid these limitations, this article set out simple and valid method to measure the concentration of the drug so that its pharmacokinetics can be studied.

Materials and Methods

Materials

Materials used in this work include plasma of Wistar Albino rat (obtained under the approval of College of Pharmacy University of Baghdad Research Ethical Committee), methanol HPLC grade (Supelco), 1-pentanol (BDH), nimodipine reference standard (EP), nifedipine reference standard (USP), and Symmetry ODS (4.6 mm x 150 mm, 5.0 µm) column (Waters).

Instruments

The main instruments that are used in this work include water bath nitrogen evaporator (Organomotion Associates, USA), centrifuge (Eppendorf, Germany), vortex mixer (Stuart, UK), freezer (Snijders Labs., The Netherlands) and HPLC system (Shimadzu, Japan),

Methods

Method development

Samples preparation

Nimodipine was dissolved in methanol to obtain stock solution from which solutions in the concentration range of 125 ng/mL to 1875 ng/mL were prepared in mixture of methanol and water (50:50v/v). Aliquots of 20 µl from of these solutions were spiked into 230 µl of rat's plasma to obtain the calibration curve and quality control (QC) solutions. To the obtained solutions, 100 µl of IS solution (nifedipine in methanol 10 µg/mL) was added, then processed by the addition of 1 mL of 1-pentanol, vortexed for two minutes, and followed by centrifugation at 4000 rpm for ten minutes. The upper layer was separated and evaporated in water bath at 35°C under nitrogen stream. The residue was reconstituted with 250 µl of the mobile phase.

Chromatography

After preparation, a volume of 100 µl from each reconstituted samples was injected into HPLC system. Chromatography was carried out at ambient temperature through ODS column with mobile phase composed of methanol:water (65:35 v/v) at a flow rate 1 mL/minute in isocratic elution. The UV wavelength was set at 238 nm. The whole procedure was performed in subdued light.

Method Validation

The developed method was validated as per the current international approach for bio-analytical method validation in order to comply with the International Conference of Harmonization (ICH) guidelines [22]. Accordingly, the following parameters were investigated.

Specificity

The specificity of the method was checked through the processing of blank plasma and injecting

the solution into HPLC system. Specificity can be confirmed by the absence of co-eluted peaks originating from endogenous plasma components at the retention times corresponding to nimodipine and nifedipine.

Linearity

Seven standard solutions using rat's plasma were prepared daily for six days to construct calibration curves. The concentration of nimodipine in these solutions which were processed as stated in the sample preparation section was 10, 20, 30, 50, 70, 100 and 150 ng/mL. The best fit, linear regression equation of peak area ratios versus concentration is used in the back-calculations, ($y = b\chi + a$), where y is the peak area ratio (nimodipine/nifedipine), χ is the concentration of nimodipine, b is the slope and a is the intercept.

Lower limit of detection and lower of limit of quantitation

The limit of detection (LOD) is the analyte's smallest concentration which can be detected, and the limit of quantitation (LOQ) is the analyte's smallest concentration which can be measured accurately and precisely by the bioanalytical method. These limits were calculated as $LOD = 3.3(SD/S)$ and $LOQ = 10(SD/S)$, where SD and S are the standard deviation and slope of the calibration curve, respectively.

Accuracy and precision

Accuracy and precision were studied over three successive days at low, medium and high concentration levels of nimodipine in QC solutions, which were calculated daily by employing the regression equation of the calibration. The deviation of the mean from nominal values measures the accuracy, while the coefficient of variation reflects the precision, both between days (inter-day) and within the same day (intra-day).

Inter-day accuracy and precision

The inter-day accuracy and precision were investigated at the three levels of nimodipine concentration (25, 60 and 125 ng/mL) in three different days. Calculations were performed for a total of eighteen QC solutions (six QC solutions per day) at each level.Intra-day accuracy and precisionThe intra-day accuracy and precision during validation were measured by analyzing six QC solutions at low, medium and high levels for nimodipine concentrations of 25, 60 and 125 ng/mL respectively within the same day; then the real concentrations were back calculated.

Recovery

The peak area signal of QC solutions was compared to peak area signal of an equivalent pure solution for each of nimodipine and nifedipine. The QC solutions were prepared through extraction. For nimodipine, recovery was calculated as the average of the three concentration levels (25, 60 and 125 ng/mL), while for nifedipine was calculated at the nominal concentration (100 ng/mL).

Stability

The stability of nimodipine in the plasma of rats which had been subjected to various storage conditions and for variable periods was studied. For this purpose, validation involved six QC solutions at each of the low and the high concentration levels (25 and 125 ng/mL) for each case.

Short-term stability

Six QC solutions at each of the two concentration levels mentioned above were analyzed to determine the initial concentration of nimodipine. Another six QC solutions were left on the bench at room temperature (25°C) for 6 hours before analysis. Short-term stability was calculated by dividing the mean concentration of QC solutions (after the stated time had elapsed) by the mean concentration of the freshly prepared QC solutions.

Long-term stability

The study of long-term stability was conducted through six QC solutions at each of the concentration levels mentioned above which were stored at (-70ºC) for 14 days then analyzed. The results of stability was calculated by the comparison between the concentrations of stored solutions with the freshly prepared ones.

Freeze-thaw stability

In this stability study, four cycles of freeze-thaw were performed. Low and high concentrations in QC solutions were prepared and stored at (-70ºC) for 24 hours then thawed unassisted at room temperature (25ºC). After complete thawing, solutions were refrozen under the same conditions. The cycle was repeated three more times, and solutions were analyzed after the fourth cycle and compared to the freshly prepared solutions.

Results and Discussion

Specificity

Peaks for nifedipine (IS) and nimodipine (analyte) were resolved as they appeared at about 6.0 and 13.8 minutes, respectively. Furthermore, the absence of any interfering peaks at these retention times indicates the specificity of the method. Both peaks of nifedipine and nimodipine were identified and well resolved (Figure 2).

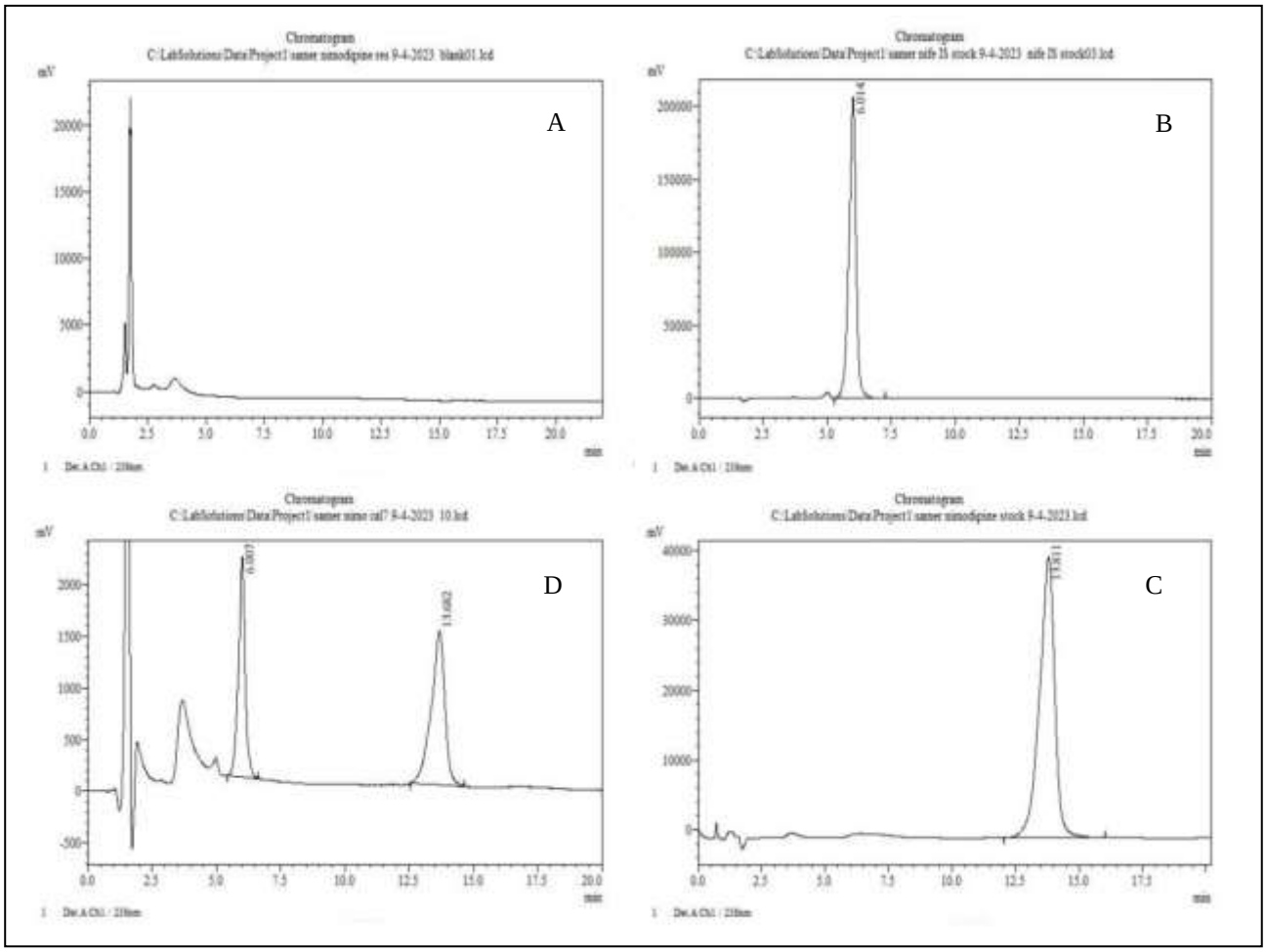

Figure 2. HPLC chromatograms for (A) processed plasma of rat as blank, (B) working nifedipine solution (IS), (C) working nimodipine solution, and (D) highest sample concentration from the calibration curve solutions (150 ng ng/mL).

The HPLC chromatograms had proved the usefulness of nifedipine as an internal standard for this method. Its benefit is obvious in quantitative bioanalysis to compen-sate for any possible loss during sample preparation, chromatography and detection [23]. Usually, a molecule with similar structure or functional groups to the analyte is chosen as internal standard because it shows similar physicochemical properties [24]. Therefore, for many drugs which share the same structural groups, it was demonstrated that optimized methods can simultaneously determine the concentrations of such drugs [25].

Linearity

The linearity of the method was assessed in the range of the constructed calibration curves (10 to 150 ng/mL) and its least squares linear regression equation. Figure 3 shows the curves for each of the six days. The regression coefficient (R^2) values span from 0.9889 to 0.9982 which indicates linear relationship between the concentration of nimodipine and the ratios of peak areas obtained from HPLC (also reflects suitable sample processing). Thus, it can be confirmed that the area under this peak is directly proportional to the concentration of analyte [26].

Lower limit of detection and lower of limit of quantitation

The average of standard deviations and slopes which were obtained from the calibration curves was utilized for the statistical calculation of LOD and LOQ [27]. The results were 0.089 and 0.269 ng/mL, respectively. These values confirmed that the concentration of nimodipine in the first calibration standard solution (10 ng/mL) well exceeded the signal to noise ratio of the peaks in HPLC chromatograms. The LOD sets to discriminate between the presence or absence of the analyte, while the LOQ depict the feasibility of the bioanalytical method to measure the analyte at very low concentrations [28].

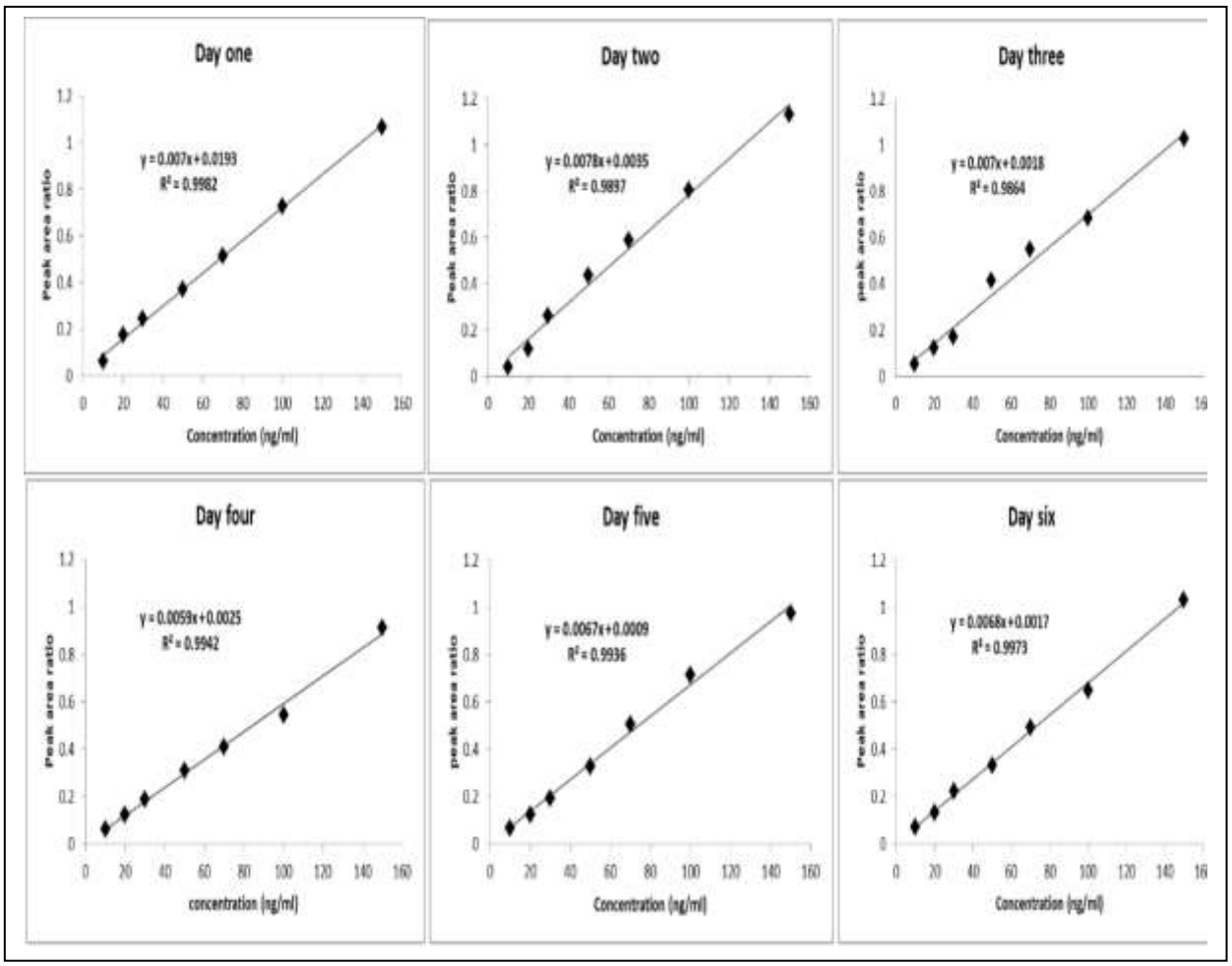

Figure 3. Calibration curves of nimodipine for six days.

Accuracy and precision

The calculated statistical parameters for QC solutions expressed the inter and intra-day accuracy and precision (**Tables 1** and **2** respectively). The concentrations of nimodipine were determined with less than 5.0% deviation from the nominal values, indicating that the method is accurate. This proves that the measured concentrations closely agree with its mean value, as demonstrated by replicating the procedure on multiple QC solutions. Such assessment helps to identify any systematic errors and assesses the recovery of the analyte [29]. The method is substantiated as precise since the coefficient of variation did not exceed 3.1% for all concentrations levels within the same day and between days of the experimental work. The results are compared for repeatedly prepared QC solutions under the same conditions within a short period of time [30].

Table 1. Inter-day accuracy and precision of Nimodipine

Nominal concentration (ng/mL)	**25**	**60**	**125**
Mean (n=18)	24.25	59.80	123.71
Standard deviation	0.542	1.221	1.233
Precision (CV %)	**2.23**	**2.04**	**1.00**
Accuracy (%)	**97.00**	**99.66**	**98.96**

Accuracy and precision verify the daily and within day repeatability of the whole procedure, covering the linear range of the bioanalytical method [31]. The stability of the analyte, the efficiency of extraction, as well as the optimized chromatographic conditions enhance accuracy, while the proper use pipettes or analytical balances, the intended minimum exposure to light or moisture, and well-trained analysts can improve precision [32].

Table 2. Intra-day accuracy and precision of nimodipine.

Working day	**within the 1st day**			**within the 2nd day**			**within the 3rd day**		
Nominal concentration (ng/mL)	25	60	125	25	60	125	25	60	125
Measured concentration (ng/mL)	23.39	63.02	122.31	24.52	62.37	122.60	25.62	58.41	123.23
	24.15	60.98	122.93	23.82	59.32	123.58	24.50	59.35	123.87
	24.08	61.06	123.08	24.47	57.18	123.12	24.48	59.34	123.35
	23.19	59.94	124.30	25.16	61.22	124.73	24.39	58.63	124.04
	23.86	59.82	126.04	24.28	59.17	126.93	23.71	58.33	123.62
	24.03	59.77	١٢٢.34	23.63	58.74	122.10	25.25	59.66	124.59
Mean (n=6)	23.78	60.77	123.5٠	24.31	59.67	123.84	24.66	58.95	123.78
Standard deviation	0.3٩٩	1.248	1.439	0.547	1.851	1.760	0.679	0.565	0.499
Precision (CV %)	**1.68**	**2.05**	**1.17**	**2.25**	**3.10**	**1.42**	**2.75**	**0.96**	**0.40**
Accuracy (%)	**95.12**	**101.28**	**98.80**	**97.24**	**99.45**	**99.07**	**98.64**	**98.25**	**99.02**

Recovery

The results comparison between the peak areas for QC solutions and those for pure solutions showed very high extraction recovery for both nimodipine and nifedipine. The data for recoveries in Tables 3 and 4 indicated that any loss for the analyte would be compensated by the internal standard. A recovery of more than 95% reflects an efficient extraction might be attributed to the suitable choice of 1-pentanal, as water-immiscible solvent for nimodipine [33]. Recovery of a bio-analytical method expresses the remaining percentage of an analyte after processing of the sample [34].

Table 3. Data of recovery for nimodipine.

Peak areas of HPLC chromatograms						
	Pure solutions			**Extracted plasma samples**		
(ng/mL)	25	60	125	25	60	125
	9187	22597	43532	8766	22579	42598
	8921	22008	45586	8693	20197	43924
	8702	21214	43068	8046	20408	41582
	9837	22123	43243	9338	21437	42450
	8822	21449	42854	8528	20478	41397
	8830	20820	43192	8627	19032	40700
Mean (n=6)	9049	21701	43579	8666	20688	42109
Recovery (%)				95.77	95.33	96.63
Average recovery				**95.91**		

On the other hand, the percentage recovery for nifedipine (86.61%) was relatively lower compared to that for nimodipine. Such percentages might be due to the difference in the partition coefficient (log P) between the two drugs [18].

Table 4. Data of recovery for nifedipine.

Peak areas of HPLC chromatograms		
	Pure solutions	**Extracted plasma samples**
(ng/mL)	100	100
	43448	37213
	42235	36390
	40504	36573
	42139	34867
	41048	35935
	40836	35729

Peak areas of HPLC chromatograms		
Pure solutions		**Extracted plasma samples**
(ng/mL)	100	100
Mean (n=6)	41702	36118
Recovery (%)		**86.61**

Stability

The stability of an analyte in biological matrix under specific storage conditions and definite time is necessary for the bioanalytical method [35].

Short-term stability

Nimodipine-processed QC solutions at low and high concentration levels gave the short-term stability data shown in Table 5. Nimodipine average concentration after 6 hours of standing at room temperature (25°C) was more than 99% close to that of fresh solutions. Besides the short period of time, the other reasons for such result are the protection of samples from light and the almost constant temperature of the laboratory [36]. The concern with dihydropyridine derivative drugs is photodegadation. Therefore, careful measures should be followed during testing and formulation of such drugs [37].

Table 5. Data of short-term stability for nimodipine.

Low QC solutions			**High QC solutions**	
Initial		6 hours	Initial	6 hours
Nominal concentration (ng/mL)	25	25	125	125
Measured concentration (ng/mL)	25.80	26.55	124.32	122.50
	25.96	26.02	124.13	122.01
	26.48	26.27	124.62	123.93
	26.02	25.91	124.09	123.06
	26.01	25.41	123.07	123.12
	25.39	25.11	123.28	122.31
Mean (n=6)	25.94	25.88	123.92	122.82
Stability (%)	**99.77**		**99.11**	
Average stability (%)	**99.44**			

Long-term stability

Pre-clinical, clinical, bioavailability or bioequivalence studies, plasma or other biological samples are usually kept frozen until the bio-analysis starts; therefore, there is prime need to check the concentration of the analyte during such storage period of time. As seen in Table 6, the average concentration of nimodipine after freezing of QC solutions at -70°C for 14 days was stable to an extent of an almost 98% compared to that of fresh solutions. Although extremely high temperatures enhance the degradation of dihydropyridine derivatives in their solid state [38], solutions of nimodipine were far more degraded through UV light than peroxide, acid, alkali or high temperature solid state [39].

Table 6. Data of long-term stability for nimodipine.

Low QC solutions			**High QC solutions**	
Initial		14 days	Initial	14 days
(ng/mL)	25	25	125	125
Measured concentration (ng/mL)	26.04	26.44	126.49	122.50
	25.55	25.18	126.53	122.69
	25.89	24.84	125.24	123.03
	25.46	24.54	126.34	123.49
	25.21	24.77	126.56	122.53
	25.78	25.38	126.04	123.41
Mean (n=6)	25.66	25.19	126.20	122.94
Stability (%)	**98.17**		**97.42**	
Average stability (%)	**97.80**			

Freeze-thaw stability

Freezing and thawing of biological samples such as plasma are stressful action. The study of nimodipine QC solutions at low and high concentrations ascertain its stability upon storage at -70ºC, then unassisted thawing for 4 cycles, resulted the data observed in Table 7. An average drug concentration of 97% was found in the QC solutions which were subjected to the temperature cycles in comparison to their initial ones. Deviations which may occur from the stated procedures may adversely affects the assay of analyte, e.g. unintended delay in handling, shipping or temporary freezing and thawing of samples [40]. Therefore, in the guidelines of bioanalytical method validation, freezing/thawing of study's solutions should mimic the conditions to which samples may be subjected during analysis. [41]. Again, photochemical degradation seems to be the most negative issue which should be minimized in order to get the correct assay for drugs like nimodipine.

Table 7. Data of freeze-thaw stability for nimodipine.

	Low QC solutions		**High QC solutions**	
	Initial	4 cycles	Initial	4 cycles
(ng/mL)	25	25	125	125
Measured concentration (ng/mL)	26.33	25.79	126.27	121.64
	26.57	25.65	126.07	121.74
	27.29	26.18	126.32	123.41
	26.83	25.55	125.89	122.16
	27.41	26.31	124.46	121.45
	27.75	27.37	125.28	122.06
Mean (n=6)	27.03	26.14	125.72	120.08
Stability (%)	**96.71**		**97.42**	
Average stability (%)	**97.06**			

Conclusion

In this work, a bioanalytical method was developed to quantify nimodipine in the plasma of rats. The method was demonstrated to be valid and thus applicable for preclinical pharmacokinetic studies of nimodipine as specific (no interference), simple (no MS detector) and fast (no need for SPE). These advantages were attributed to the suitable selection of the solvent for extraction as well as the verified choice of chromatographic conditions with effective UV detection at very low concentrations. Therefore, this method precludes the need sophisticated techniques e.g. MS detection. Other extraction solvents and IS were utilized in the previously published methods for nimodipine. The protection of samples from direct exposure to light upon handling should be considered to render the stability of the analyte and IS. As rule of thumb, standard calibration curve must be generated with each analytical run for unknown samples. Finally, QC solutions at several concentration levels should be prepared and analyzed simultaneously so that any analytical drift can be detected and the results are correctly judged.

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Conflicts of Interest

This article is submitted by the authors who clearly declare that they do not have any conflict of interest with any scientific and/or commercial organization.

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Ethics Statements

The plasma of Wistar Albino rat was used after the College of Pharmacy, University of Baghdad Research Ethics Committee issued the certificate with an approval number REAFUBCP122023A.

Author Contribution

The authors confirm contribution to the paper as follows: study conception, design, experimental work and data collection by Samir Hasson Aziz Ramadhan. Analysis and interpretation

of results by Samir Hasson Aziz Ramadhan and Khalid Kadhem Al-Kinani. Draft manuscript preparation: Samir Hasson Aziz Ramadhan. Both authors reviewed the results and approved the final version of the manuscript.

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تطوير و تقييم طريقة لقياس تركيز نيموديبين في بلازما الجرذان

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الخلاصة

تم تطوير طريقة تقييم كمي لنيموديبين في بلازما الجرذان بإستخدام منظومة الكروماتوغرافيا السائل عالي الأداء و تقييم صلاحية الطريقة و تتميز الطريقة بإستخدام ١- بينتانول كمذيب في الإستخلاص من بلازما الدم عند تحضير العينات ، و كذلك أستخدم نيفيديبين كمعاير داخلي أثناء عملية الفصل الكروماتوغرافي خلال عمود نوع أوكتاديسيل سيلان مع طور متحرك يتكون من الماء و الميثانول. تم إثبات صلاحية الطريقة من نواحي الخصوصية مع المدى الخطي من ١٠ إلى ١٥٠ نانوغرام نيموديبين لكل مليلتر. نتائج التقييم كانت كالأتي: الدقة بين الأيام و في ذات اليوم تتراوح من ٩٧,٠٠ إلى ٩٩,٦٦٪ و من ٩٥,١٢ إلى ١٠١,٢٨٪ على التوالي ، الإنضباط بين الأيام و في ذات اليوم يتراوح من ١,٠٠ إلى ٢,٢٣٪ و من ١,١٧ إلى ٣,١٪ على التوالي. نسبة الإستعادة لكل من نيموديبين و نيفيديبين وجدت ٩٥,٩١٪ و ٨٦,٦١٪ على التوالي. أخيرا ، كانت نتائج الثباتية للفترة القصيرة في درجة حرارة الغرفة ٩٩,٤٤٪ و للفترة الطويلة في درجة التجميد ٩٧,٨٪ أما في حالة التجميد ثم الإسالة ٩٧,٠٦٪ . يمكن الإستنتاج في ضوء هذه النتائج إلى صلاحية هذه الطريقة و إمكانية تطبيقها في تحليل العينات المستخدمة في دراسات حركية الدواء.

الكلمات المفتاحية: طريقة تحليل لعينات حياتية، الكروماتوكرافي السائل عالي الأداء، نيموديبين، حركية الدواء قبل السريرية، تقييم صلاحية