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Development and validation of a green RP-HPLC method for detection and quantitation of meropenem trihydrate in the bulk: A comparison with HPTLC method

Mais Bashimam[a*] and Hind El-Zein[a]

[a]*Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy, Damascus University, Syrian Arab Republic*

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A B S T R A C T

This research introduces an eco-friendly green Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) method for detecting and quantifying meropenem trihydrate. Traditional methods use buffered solutions, gradient mobile phases, and longer retention times, but this method offers a rapid RP-HPLC technique with a C8 column and isocratic mobile phase (60% methanol, 40% ultra-pure water), eliminating the need for buffers and acetonitrile. It features a short 2.1-minute retention time and a high r^2 value of 0.9995. It delivers accuracy (101.1-102.3%) and precision (RSD ≤ 2%), coupled with low LOD and LOQ values of 1.72 and 5.20 µg/ml. Aqueous dilution simplifies sample preparation, reducing degradation and interference. The method is compared with an HPTLC method, showing an extended linear range (6.25-200 µg/ml for HPLC, 7.81-62.5 µg/ml for TLC) and high sensitivity, making it significant for meropenem trihydrate quality control in bulk and dosage forms.

1. Introduction

Meropenem trihydrate (MER) is a broad-spectrum antibiotic that belongs to the carbapenem class. It exerts its antimicrobial activity by inhibiting bacterial cell wall synthesis; binding to and hindering the activity of penicillin-binding proteins (PBPs), which are responsible for cross-linking the peptidoglycan strands in the bacterial cell wall, leading to bacterial death. [1] MER is effective against both Gram-positive and Gram-negative bacteria, including those that are resistant to other antibiotics. [2] **Fig. 1** shows the chemical structure of MER. The pyrroline ring provides additional stability to the beta-lactam ring, making it less susceptible to hydrolysis by beta-lactamases when compared to other beta-lactam antibiotics that have a thiazolidine ring. [3]

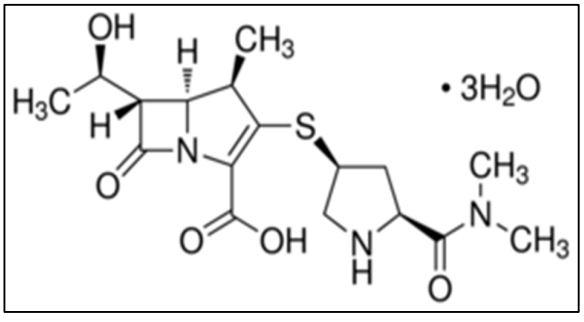

Fig. 1. The chemical structure of meropenem trihydrate (MER).

* Corresponding author.
E-mail address mais.bashimam@damascusuniversity.edu.sy (M. Bashimam)

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There have been reports in the literature regarding the instability of MER under certain conditions. For example, Mendez *et al*. found that MER degraded more rapidly in solution at higher temperatures and pH values above 8.0. [4, 5] Another study in 2013 found that MER degraded significantly when exposed to light and organic solvents. [6]

MER is available in injectable formulations because it is not well absorbed when taken orally. Oral absorption of MER is low due to its hydrophilic nature and susceptibility to degradation by enzymes in the gastrointestinal tract. [2]

Although pharmacopoeias contain compendial methods for assay, many procedures for MER quantification analysis were reported and published. For example, a comparative review by Cielecka-Piontek *et al*. (2011) summarized all the used analytical techniques to its date. [7] Later, many methods were developed, validated and published to fit different analytical situations; i.e., substance state, dosage form and excipients, [8, 9] examined media (blood serum, cerebrospinal fluid, aqueous solution). [1,10,11] Yet, there will always be a need to develop new methods with different conditions according to the available resources and apparatuses.

Validation assessments are crucial when adopting a new analytical method, and international guidelines exist to ensure the reliability, accuracy, and suitability of these methods. Although ICH, FDA, and USP have slight differences in their guidelines, the core principles and requirements for analytical method validation are generally similar. ICH Q2(R2) offers guidance on validation, emphasizing characteristics like accuracy, precision, specificity, and linearity. It also covers validation plan development and documentation.[12] FDA's guidance focuses on ensuring safety, efficacy, and quality of pharmaceutical products through analytical procedures and methods validation.[13] In contrast, USP's General Chapter <1225> outlines the validation process and recommends evaluating performance characteristics, emphasizing robustness testing, and employing experienced analysts, proper equipment, and procedures. [14] Column C8 was used during our research, however, this transition introduced complexities. Many of the methodologies associated with the C8 column involve using a mobile phase in gradient mode and employing buffer solutions that lead to elevated pressure levels within the column and therefore decreasing its lifetime. On the other hand the use of acetonitrile in most cases is not considered an eco-friendly option. [15, 16] This path ultimately led us to explore the validity of the developed RP-HPLC method according to the ICH Q2(R2) guidelines, by using mixtures of methanol and water as a mobile phase in addition to dissolve both the standards and samples of the bulk powder in water instead of buffered solution.

2. Results and Discussion

2.1. System Suitability

After numerous attempts to achieve a sharp peak with a stable retention time, it was discovered that currently available pharmacopeial and published methods, which involve high ratios of buffered aqueous phase containing salts, acids, and bases along with a small ratio of organic solvent, did not yield satisfactory results. [7,11] These mobile phases led to excessive column pressure exceeding 170 bar, retention times of over 10 minutes, and imperfect peak shapes. In light of this, the HPTLC technique described by Farid and Abdelwahab was explored, [17] but it had limitations in terms of linearity range and the potential instability of MER upon dilution with methanol. To find a suitable chromatographic condition, the focus shifted to molecules with similar solubility and polarity to MER. Eventually, a method utilizing a C8 column and a mobile phase consisting of 55:45 v:v methanol-water, previously employed for phenytoin, [18] was attempted and yielded the desired outcome. The optimization of the mobile phase ratio aimed for 60:40, resulting in a column pressure of 80-100 bar and a sharp peak retained in approximately 2 minutes. System suitability tests are essential to assess the reliability of chromatographic methods, and to ensure that the chromatographic system performs optimally. [19] Retention time, number of theoretical plates, resolution, capacity factor, and tailing factor were analyzed for five consecutive injections of a drug solution with a concentration of 50 µg/ml, and the obtained results were within acceptable limits, as shown in **Table 1**, MER contents are expressed as means ± standard deviation. [20]

Table 1. Suitability test parameters for meropenem trihydrate (MER).

Parameter	Values for MER ±SD	Acceptance Criteria
Theoretical Plates Count	39282.8 ± 356.83	> 2000
Tailing Factor	0.89 ± 0.01	≤ 1.5
Capacity Factor	27.55 ± 1.25	> 2
Retention Time	2.05 ± 0.02	-
Resolution	2.63 ± 0.35	> 2

2.2. Linearity

A linear relationship of the peak area as a function of concentration was confirmed. For the calibration curve, six concentrations of MER (6.25, 12.5, 25, 50, 100, and 200 µg/mL) were measured in replicate (n = 3). The mean standard calibration curve was established through a least square linear regression model. Correlation of determination (r2 = 0.9995) was obtained, which meets with the acceptance criteria of not less than 0.999. Therefore, it was concluded that the method is linear. Further unknown determinations are concluded from the resulting curve equation. The relative standard deviation (RSD%) for the three replicates of MER standard solution is less than 2.0% as shown in **Table 2**. A typical chromatogram

for MER peaks of five standard series concentrations (6.25, 12.5, 25, 50 and 100 ppm µg/ml) is shown in **Fig. 2-A** where retention time Rt = 2.15 min, and Calibration curve of MER with equation and correlation of determination in **Fig. 2-B**.

Table 2. Mean and RSD% of area under the peak (AUC) as a factor of standard serial concentrations of meropenem trihydrate MER.

Concentration (µg/ml)	AUC (m.AU.s)			Mean	RSD%
6.25	166.28	163.26	170.404	166.65	1.76
12.5	339.67	336.49	344.745	340.30	1.00
25	665.56	673.103	676.535	671.73	0.68
50	1360.971	1367.975	1339.234	1356.06	0.90
100	2541.411	2664.016	2590.763	2598.73	1.94
200	5062.174	5177.545	5185.708	5141.81	1.10

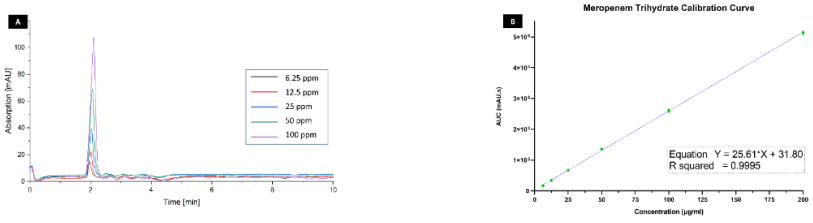

Fig. 2. (A) HPLC chromatogram of MER peaks of six standard series concentrations (6.25, 12.5, 25, 50 and 100 ppm µg/ml), Rt = 2.15 min. (B) Calibration curve of MER with equation and correlation of determination.

2.3. Precision and Accuracy

The precision of the method was verified by inter-day and intra-day variation studies. In the intra-day studies, three repeated injections of three standard solutions (25, 50, 100 µg/ml) were made and the area under MER peak and RSD% were calculated (**Table 3).** While in the inter-day variation studies, the same procedure was made for three distinct days and response factors of drug peak and RSD% were calculated as shown in **Table 3.** From the data obtained, the developed method was found to be precise as RSD% was not more than 2.0%. The method is also accurate because the percentage recovery for MER at 25, 50, 100 µg/ml (in replicates for each concentration n = 3) ranges from 101.1 to 102.2% which is well within the acceptance criteria of 95.0 to 105%. (RSD ≤ 2%), as shown in **Table 3**.

Table 3. Precision and Accuracy results for method validation

Theoretical concentration (µg/ml)	Accuracy (%)	Precision			
		Inter-day ± SD (Intermediate)	RSD %	Intra-day ± SD (Repeatability)	RSD %
25	101.1	25.26 ± 0.54	2	21.72 ± 0.31	1.4
50	102.2	51.08 ± 1	2.0	46.39 ± 0.44	0.9
100	101.3	101.26 ± 2.37	2	93.75 ± 0.92	1.0

2.4. Specificity

To evaluate the specificity for the developed analytical method, the resolution between MER peak and a product of degradation peak was studied. The British Pharmacopeia suggests to study degradation products for MER by forcing a sample solution to degrade by heating to 60 °C for 20 minutes [21]. But to make sure MER was fully degraded, a stock solution of 200 µg/ml MER was heated in a boiled water bath (100 °C) for 2 hours. Then a proper dilution was made to obtain a

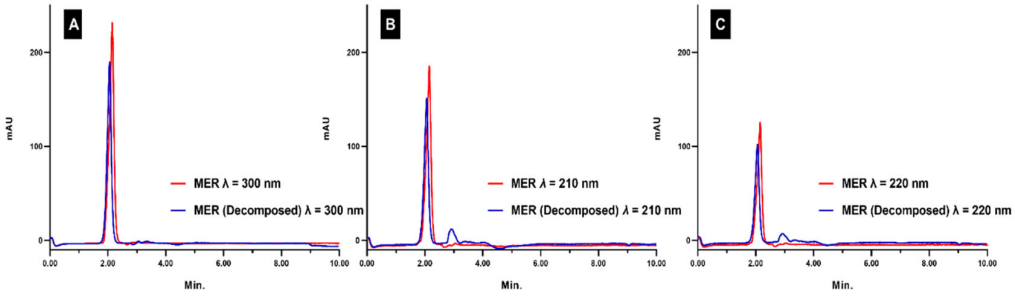

Fig. 3. Specificity study chromatograms. Red line represents the meropenem trihydrate MER sample (100 µg/ml) and the Blue line represents the decomposed MER by heating (100 µg/ml) detected at different wavelengths: (A) chromatogram at λ= 300 nm, (B) chromatogram at λ= 210 nm, and (C) chromatogram at λ= 220 nm.

sample of MER (100 µg/ml) which contains degradation results. The sample was analyzed using wavelength 210 nm for detecting decomposition results. The resulted chromatogram contains two peaks; MER peak (Rt = 2.06 min) and decomposition product peak (Rt = 2.92) (**Fig. 3-B).** The wavelength of 300 nm cannot detect decomposition product as shown in **Fig. 3-A.** It is important to mention that the British Pharmacopoeia monograph of MER detects degradation products using UV wavelength = 220 nm, but when optimizing the chromatographic conditions in our lab the absorbance response in 210 nm was higher than 220 nm **(Fig. 3-C).** So, UV wavelength of 210 nm was applied. **Table 4** clearly shows that the developed method has good specificity because the obtained results were within acceptable limits.

Table 4. Specificity study for meropenem trihydrate MER and its heat-decomposition product

Parameter	Values for MER	Values for Decomposition result	Acceptance Criteria
Theoretical Plates Count	36734	26967	> 2000
Tailing Factor	0.871	1.3	≤ 1.5
Capacity Factor	24.7	35.5	> 2
Retention Time	2.06	2.92	-
Resolution	--	2.39	> 2

2.5. Limit of Detection and Limit of Quantification (Sensitivity)

The lower range limits (Limit of Detection LOD and Limit of Quantitation LOQ) can be assessed using standard deviation of linear regression and the slope applying the equations mentioned in Methods. Hence, LOD and LOQ are 1.72 and 5.20 µg/ml, respectively.

2.6. Robustness

The robustness of the method is a key characteristic that must be evaluated during the method. It is useful in order to study the effects of method parameters on the analytical procedure. [12] In this study, we assessed the robustness of our method by subjecting it to mild variations in mobile phase composition (methanol: water 55:45 and 60:40) and column temperature (30 °C and 40 °C), using a standard solution (200 µg/ml) as the test sample. The results are summarized in **Table 5** within the acceptance criteria of 95.0 to 105%. Our findings suggest that the method remains reliable under these conditions, with minimal impact on the analytical performance.

Table 5. Robustness of the method results

		Rt (min.)	Accuracy * (%)
Mobile Phase	55:45	1.95	100.5
	60:40	2.15	95.8
Column Temperature	25°	2.21	97.4
	40°	2.15	95.8

* The acceptance criterion is 95.0 to 105%.

2.7. High Performance Thin Layer Chromatography method (HPTLC)

By applying Farid and Abdelwahab validated methods as mentioned earlier in Methods, [17] a stock solution of MER has a concentration of 1 mg/ml in methanol using 10 ml measuring flask. After a proper dilution with methanol, a standard series of MER was obtained with concentrations of: 3.91, 7.81, 15.625, 31.25, 62.5, 125, 250 and 500 µg/ml a 3D chromatogram is shown in **Fig. 4** which reveals that the method can detect concentrations from 7.81 to 62.5 µg/ml, over that it needs dilution. **Fig. -A** represents a 2D chromatogram of standard concentration of MER (62.5 µg/ml) with a retention factor Rf = 0.18 (Farid's reported Rf is 0.19). By plotting the maximum height of peaks as a factor of concentration, a calibration curve is obtained as shown in **Fig. -B.** The curve is linear (R^2 = 0.9961) in the narrow range of concentrations between 7.81 and 62.5 µg/ml. Although the HPTLC method is linear, the detecting range is not wide enough to detect MER practically.

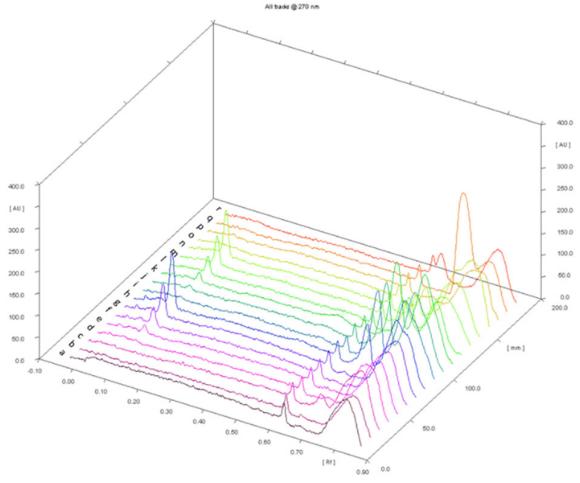

Fig. 4. HPTLC 3D chromatograms of standard serial concentrations of meropenem trihydrate MER in methanol: (a-b-c) methanol, (d) 3.91 µg/ml, (e) 7.81 µg/ml, (f) 15.625 µg/ml, (g) 31.25 µg/ml, (h) 62.5 µg/ml, (i - r) a replicate for the standard series and blanks.

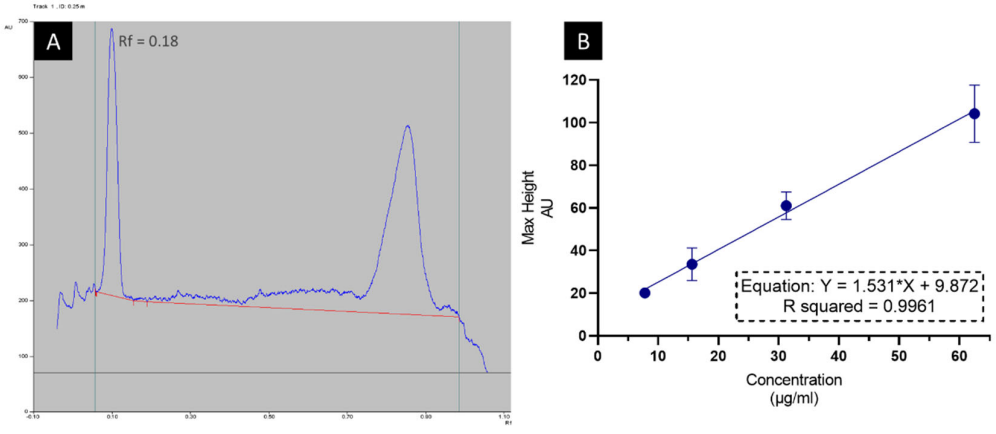

Fig. 5. (A) HPTLC 2D chromatogram of MER standard solution (62.5 µg/ml) with Relative Factor (Rf) = 0.18. (B) Calibration curve of the HPTLC method (R^2 = 0.9961)

3. Conclusion

A Green analytical method has been developed and validated for the detection and quantification of MER in the bulk and in the presence of degradation products using RP-HPLC with a C8 column. The method is reproducible, specific, precise, and accurate. It is a simple technique that can be applied in every laboratory equipped with a basic HPLC system. Furthermore, this HPLC method is an improvement over the HPTLC method reported in the literature due to its wider linear range and the use of aqueous dilution in addition to less organic solvents, by using 60% methanol and 40% ultra-pure water, thereby eliminating the need for buffer solution and acetonitrile. The method has a short 2.1-minute retention time and a high r^2 value of 0.9995. It is accurate (101.1-102.3%) and precise (RSD ≤ 2%), coupled with low LOD and LOQ values of 1.72 and 5.20 µg/ml. The wider linear range of this method allows for more accurate and precise quantification of the target analyte in addition to be a stability indicator, while the use of aqueous dilution simplifies the sample preparation process and minimizes the risk of degradation and interference from impurities.

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4. Materials and Methods

4.1. Materials

Meropenem trihydrate was purchased from Qilu Antibiotics (Linyi Pharmaceutical Co., Ltd., Shandong, China). HPLC-grade methanol, Ethyl acetate and Formic acid were purchased from Merck (Germany). All aqueous solutions were prepared with ultra-pure water purified using Easy pure II system, Barnstead International (USA).

4.2. Chromatographic Apparatus and Conditions

The HPLC system (Sykam, Germany) was equipped with Reagent organizer (S7131), a solvent delivery system (S2100), auto injector (S5200), a column thermocontroller (S4011), and a UV/VIS Multichannel detector (S3240). The chromatographic analysis was performed using an analytical Inert Sustain C8 HPLC Column, 5 µm, 250 x 4.6 mm (GScience, Japan). The mobile phase was a mixture of methanol and ultra-pure water (60:40, v:v), degassed in a digital ultrasonicater (Branson 1210 Bransonic, USA) for 10 minutes without further filtration. The analyses were performed at a flow rate of 1 ml/min isocratically. The column was thermostated at 40 °C. MER was detected at 300 nm quantitively and 210 nm for decomposition products, qualitatively. Volume of the injection was set to 20 µl. The total time between injections was 10 min. All retrieved Data were processed using *Clarity* software (v. 8.2.0.78, Datapex Ltd, 2019).

4.3. Standard Solution Preparation

A 200 µg/ml stock standard solution of MER was prepared by dissolving an accurately weighed 2.00 mg of MER in 5 ml of ultra-pure water in a clean dry 10 ml volumetric flask, followed by vortex agitation in mini-shaker till complete dissolving, then completing the volume to 10 ml with ultra-pure water and shake for homogeneity. Ultra-pure water was used as a diluent in all stocks and samples analyses.

4.4. Method Validation

The methodology was tested for validation parameters according to the international Council for Harmonization (ICH) of Technical Requirements for Pharmaceuticals for Human use Guideline, [12] including system suitability, linearity, specificity, precision, accuracy, limit of detection, limit of quantification and robustness.

4.5. System suitability

System suitability was tested by five replicate analyses of MER solution with a concentration of 50.0 µg/ml by evaluating chromatographic parameters including theoretical plates, capacity factor, and tailing factor. A calculation of the mean and relative standard deviation (RSD%) was performed.

4.6. Linearity

The calibration curve of MER is established by analyzing a series of concentration of the stock solution of MER and making the suitable dilutions to obtain six points of concentrations: 6.25, 12.5, 25, 50 and100, in addition to 200 µg/mL (in triplicate), and plotting the Area Under the Curve (AUC) as a function of MER concentration (*X*). Linearity is calculated by linear regression equation (1):

$$AUC = (Slope \times X) + Intercept \quad (1)$$

RSD% of the slopes and the intercepts are determined, and a correlation of determination (r^2) of more than 0.999 must be detected.

Further unknown determinations are concluded from the resulting equation.

4.7. Precision and Accuracy

Intra-day variability is assessed by replicate analysis of the calibration standards in the same day, whereas inter-day variability is measured by replicate analysis of the calibration standards in different days (one replicate each day). Accuracy of the method is determined by comparing practical recovered amounts with actual values present in the samples (theoretical values). Precision is tested by evaluating the relative standard deviation (RSD%) for each sample which is recommended to be less than 2%.

4.8. Specificity

Specificity of the developed HPLC method is estimated by studying resolution between MER peak and all nearest peaks that could interfere with quantification. To obtain another peak in the chromatogram, a stock solution (200 µg/ml) of MER is boiled in water bath for 2 hours. Then a sample of 100 µg/ml aqueous solution is prepared by proper dilution of the decomposed stock and analyzed.

4.9. Limit of Detection and Limit of Quantification (Sensitivity)

In order to determine the minimum concentrations which can be identified and measured via the developed method, the slope and standard deviation of a linear regression can be used to evaluate the lower range limitations (Limit of Quantitation LOQ and Limit of Detection LOD) using the following Eq. (2) and Eq. (3):

$$LOD = \frac{3.3\,\sigma}{S} \tag{2}$$

$$LOQ = \frac{10\,\sigma}{S} \tag{3}$$

where σ = the standard deviation of the response

S = the slope of the calibration curve

Based on the calibration curve (in triplicate), σ can be determined by the standard deviation of y-intercepts of the regression lines and S is the slope of the calibration curve.

4.10. Robustness

Assessing the robustness of a method during validation is crucial, as it allows for the examination of the effects of varying method parameters on the analytical procedure.[13] In this study, the robustness of the method was evaluated by subjecting it to mild variations in mobile phase composition (methanol: water 55:45 and 60:40) and column temperature (30 °C and 40 °C) using a standard solution (200 µg/ml) as the test sample.

4.11. High Performance Thin Layer Chromatography method (HPTLC)

As reported in the work of Farid and Abdelwahab (2019), their validated HPTLC method was applied in our work to compare the feasibility and practicality between RP-HPLC and HPTLC analysis. The used stationary phase was TLC aluminum plates (20 x 10 cm) pre-coated with silica gel 60 F_{254} with 200 µm thickness and 5 µm particle size (Merck, Darmstadt, Germany). CAMAG Automatic TLC Sampler4 was used for sampling. Samples were sprayed as bands using a Linomat V applicator with 25 µl syringe. Band length: 6 mm. Volume: 20.0 µl. Solvent front position 95 mm. Scanning was done by CAMAG TLC Scanner 3 (Camag, Muttenz, Switzerland) controlled with winCATS software (version 1.4.4.6337). Detection was carried out using wavelength 270 nm. Lamp: D2.

The mobile phase consisted of a solvent mixture of ethyl acetate: methanol : deionized water : formic acid (60:30:15:1, by volume). Using a glass jar, the chromatographic development was done after saturating with the mobile phase mixture for 15 min. The temperature was maintained constant at 25° C. [17]

Stock solution of 1 mg/ml was prepared in methanol using 10 ml measuring flask and a proper dilution with methanol was used to conduct a standard series of MER (3.91, 7.81, 15.625, 31.25, 62.5, 125, 250, 500 µg/ml).

4.12. Statistical Analysis

GraphPad Prism (v. 9.5.0) for Windows (GraphPad Software, San Diego, California USA) was used for graphing and interpolating unknowns from calibration curves. Mean ± SD and RSD% was calculated using MS Excel (Microsoft Office Professional Plus 2019). This work confirms the high importance of organic molecules due to their various applications as reported before.[22-27]

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