Method development for the determination of metformin in human plasma by capillary zone electrophoresis

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Abstract

Background: Metformin is an oral antidiabetic drug from the biguanide class and it is the first-line drug chosen for the treatment of type 2 diabetes. This is a drug on the list of pharmaceutical substances required to report bioequivalence study data when registering the drug. **Objectives**: (1) To develope a capillary zone electrophoresis method for determining metformin hydrochloride in human plasma. (2) To validate the method. **Materials and methods**: Human plasma, metformin hydrochloride, ranitidine hydrochloride. The method was developed and validated according to US-FDA 2018 and EMA 2011. **Results:** The procedure was developed by using the Agilent 7100 CE electrophoresis system with ranitidine hydrochloride as an internal standard. Sample preparation was accomplished through protein precipitation with acetonitrile. The optimal electrophoresis conditions are as follows: uncoated fused-silica capillary column of a total length of 40 cm (31.5 cm effective length, inner diameter 100 µm), phosphate buffer solution 100 mM (pH = 4), the voltage applied to both capillary ends of 15 kV, the inlet end of capillary dipped in water before sample injection, sample injection mode of 50 mBar in 7 seconds, using a PDA detector at 232 nm. The analysis method was validated according to the requirements of the US-FDA 2018 and EMA 2011 with the following criteria: system suitability with RSD < 3%; good specificity; the calibration curves were linear ($r^2 \geq 0.98$) in the concentration range of 0.1 – 4.0 µg/ml for metformin in human plasma; the lower limit of quantification was 0.1 µg/ml; the intra-day and inter-day accuracy were 99.41 – 105.28% and 92.47 – 106.26%, respectively; the intra-day and inter-day precision were 1.99 – 4.03% and 3.11 – 6.05%, respectively; the mean recovery of ranitidine (internal standard) was 86.2%, the mean recoveries of metformin at three levels LQC, MQC, and HQC were 72.9%, 75.9%, and 77.4%, respectively; plasma samples were stable to analysis. **Conclusions**: The developed method meets the requirements of US-FDA 2018 and EMA 2011 to determine metformin in plasma.

Keywords: *Capillary zone electrophoresis, plasma, metformin*

1. BACKGROUND

Metformin is a biguanide-class oral antidiabetic drug with a different mechanism of action than other antidiabetic drugs [4]. It belongs to group III in the biopharmaceutics classification system with high solubility in water and poor permeability to cell membranes, which means that the preparation technique has a significant impact on the drug's bioavailability. Therefore, metformin is a drug that require *in-vivo* bioequivalence testing when registering a generic product [7]. For this reason, it is necessary to validate metformin analytical procedures in biological fluids (usually in plasma) in accordance with guidelines of US-FDA 2018 and EMA 2011 [9, 14].

In the world, there have been a number of studies on the quantification of metformin in human plasma using various methods, with the most widely used methods being high - performance liquid chromatography (HPLC) [6, 10, 11], and capillary electrophoresis (CE) [12, 13, 15].

In Vietnam, metformin in human plasma was measured by high-performance liquid chromatography [1, 2, 5]. However, up to now, there has been no study published on the quantitative determination of metformin in biological fluids by capillary electrophoresis.

This analytical method has many advantages such as high separation performance, short analysis time, saving consumable supplies. In order to develop an applicable method for quantifying metformin in human plasma, we conduct the study to develop a capillary electrophoresis method.

2. MATERIALS AND METHODS

2.1. Materials

Human plasma was supplied by the National Institute of Hematology and Blood Transfusion.

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Secondary standards, metformin hydrochloride (99.12%) and ranitidine hydrochloride (98.36%) were purchased from the National Institute of Drug Quality Control.

All other chemicals used in the study were sodium dihydrogen phosphate (NaH_2PO_4), acid orthophosphoric (H_3PO_4), sodium hydroxide (NaOH), acetonitrile, methanol (MeOH, Merck, Germany), and double-distilled deionized water.

Instrumentation – equipment: Agilent 7100 capillary electrophoresis system, HI 2550 - 02 pH meter (Hanna, Italy), double distilled water machine A400D (UK), vortex mixer VX – 200 Labnet (USA), centrifuge Labnet Spectrafuge 24D (USA), analytical balance HR-250AZ (Korea), refrigerator preserved sample TOSHIBA (Japan), elmasonic S100H ultrasonic cleaner (Germany), fused – silica capillaries Agilent Technology (USA); Glassware: volumetric flasks type 10 ml, beakers, micropipettes,...

2.2. Methods

2.2.1. CE Method Development

Preparation of standard solutions

Standard stock solutions were prepared by dissolving the metformin standard in distilled water to obtain an exact concentration of about 100 µg/ml.

The internal standard solution was prepared by dissolving the ranitidine standard substance in water with an exact concentration of about 100 µg/ml.

From stock standard solutions, working standard solutions (QC1, QC2) in plasma were prepared with metformin concentrations of about 4.0 µg/ml and 1.0 µg/ml, respectively.

Dilute QC1, QC2 with blank plasma to obtain standard samples with metformin concentration from 0.1 to 4.0 µg/ml to prepare samples to build standard curves.

Dilute QC1, QC2 with blank plasma to obtain test samples including 3 different concentrations (LQC 0.3 µg/ml, MQC 2.0 µg/ml, HQC 3.0 µg/ml).

Sample preparation

The protein precipitation method [1, 2, 5] and the liquid-liquid extraction method [6] were conducted.

Electrophoretic conditions

Fixed electrophoretic conditions are as follows: uncoated fused-silica capillary column of a total length of 40 cm (effective length 31.5 cm) [13]; the capillary temperature was set at 25^0C [13]; injection pressure was set at 50 mBar [12, 13], the wavelength of detection was set at 232 nm [15].

The preliminary studies were conducted to select capillary column diameter, buffer solution type, buffer solution concentration, buffer solution pH, the voltage on capillary ends, and sample injection time.

2.2.2. Method validation

The method was validated according to US-FDA 2018 and EMA 2011 about the validation of analytical procedures in human plasma including the following criteria: system compatibility, specificity, linearity range, the lower limit of quantitation, precision, accuracy, recovery rate (extraction efficiency); stability.

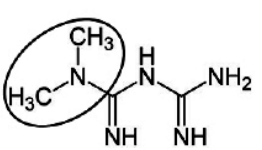
Data were expressed as the mean ± standard deviation, calculated using Microsoft Excel 2016 software.

3. RESULTS

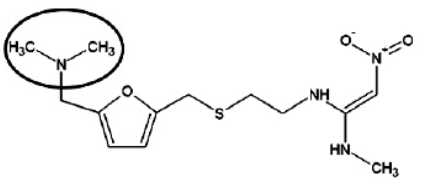
3.1. CE method development

3.1.1. Selection of internal standard

Ranitidine hydrochloride was chosen as the internal standard in this study because it has a dimethylamino group similar to that of metformin.



Metformin



Ranitidine

3.1.2. **Sample preparation**

Protein precipitation methods were conducted with two protein precipitation agents, including acetonitrile or perchloric acid 70% (w/v). By using perchloric acid, metformin were not separated from the matrix, while by using acetonitrile, metformin and ranitidine were separated from the matrix, and peak responses were stable. Therefore, acetonitrile was selected as the protein precipitation agent.

After selecting the protein precipitation agent, experiments were conducted to choose the proper centrifuge time among 5 minutes, 10 minutes, and 15 minutes at 10000 rpm. The results showed that after centrifugation for 5 minutes, the sample matrix was

not clean, the extraction efficiency was low (46.69%); after centrifugation for 10 minutes, the sample matrix was relatively clean, the extraction efficiency was quite high (73.50%); after centrifugation for 15 minutes, the matrix was also clean and the extraction efficiency was lower than that of 10 minutes (58.80%). Therefore, the centrifuge time of 10 minutes was selected for further analysis.

Finally, the sample preparation process was conducted as follows: 50 µl of ranitidine hydrochloride solution 40 µg/ml (internal standard) and 1 ml of acetonitrile were added to 1 ml of plasma samples (blank/standard) in a 5 ml centrifuge tube, vortexed for 2 min then centrifuged for 10 min at 10000 rpm. The supernatant layer was filtered through a 0.45 µm membrane filter. The filtrate (1 ml) was transferred to another 1.5 ml Eppendorf centrifuge tube, evaporated to dryness with a centrifugal evaporator. The residues were reconstituted with 100 µl of a mixture of acetonitrile and water (1:1, v/v), vortexed for 30s before analysis.

3.1.3. Electrophoretic conditions

The preliminary studies were conducted to select CE conditions that provide good separation and good peak shape. The electrophoretic separation was achieved on an uncoated fused-silica capillary column (total length 40 cm, effective length 31.5 cm, inner diameter 100 µm), maintained at 25^{0}C. The detection wavelength was set at 232 nm. Phosphate solution (100 mM, pH = 4) was used as the buffer. The applied voltage was set at 15 kV. Before injecting samples, the inlet end of the capillary was dipped in water. Sample injection mode was set at 50 mBar in 7 seconds.

3.2. Method validation

3.2.1. System suitability

Capillary electrophoresis system suitability was determined by repeated injection of 6 standard solutions of metformin at the concentration of 2.0 µg/ml. Results of the capillary electrophoresis system suitability are presented in *Table 1*.

Table 1. Results of capillary electrophoresis system suitability (n = 6)

Serial Number	Metformin				Ranitidine				R_S
	T_R *(min)*	S *(mAu.s)*	N	A_s	T_R *(min)*	S *(mAu.s)*	N	A_s	
1	2.097	182.8	7158	1.1	2.648	63.5	4804	1.1	4.4
2	2.098	184.2	7158	1.0	2.669	63.1	5127	1.1	4.6
3	2.156	184.3	7361	1.0	2.757	63.1	5328	1.2	4.8
4	2.134	187.9	7027	1.0	2.733	64.1	4999	1.2	4.7
5	2.128	184.2	6609	1.1	2.737	65.4	4652	1.2	4.6
6	2.140	187.0	6877	1.0	2.736	63.4	5385	1.2	4.7
Mean	2.126	185.1	7032	1.0	2.713	63.8	5049	1.2	4.6
RSD (%)	1.11	1.05	3.72	5.00	1.62	1.38	5.72	4.43	2.95

T_R: Retention time; S: Peak area; N: Plate theory; R_S: Resolution; A_S: Asymmetry

According to *Table 1*, the relative standard deviations of retention time and peak area are within the acceptance range (< 3.0%); 0.8 ≤ As ≤ 1.5; Rs ≥ 1.5 [3]. This showed that the capillary electrophoresis system is suitable for the determination of metformin in human plasma.

3.2.2. Specificity

Analyze 6 blank plasma samples and 6 standard solutions of metformin 0.1 µg/ml in plasma. Electropherograms are shown in *Figure 1* and *Figure 2.*

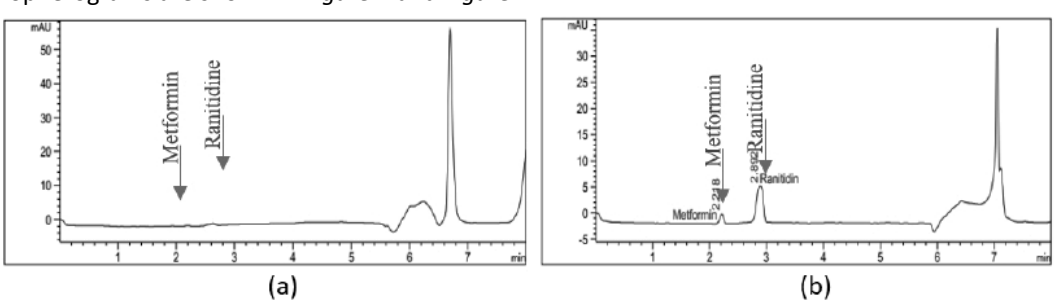
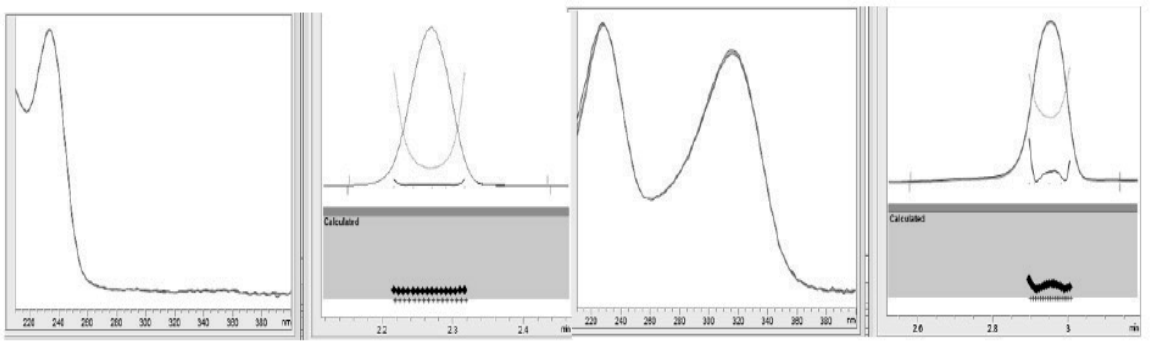


Figure 1. Electropherograms of (a) Blank plasma sample; (b) Standard plasma sample spiked with metformin and ranitidine



(a) (b)

Figure 2. UV spectrum and peak purity
(a) Metformin; (b) Ranitidine

In the electropherogram of standard solutions, 2 peaks of metformin and ranitidine were detected at 2.2 minutes and 2.9 minutes, respectively (*Fig. 1b*); whereas peaks of metformin and ranitidine were not found in the blank at those retention times *(Fig. 1a)*. The results of UV spectroscopy tests of two peaks using a PDA detector *(Fig. 2)* showed that these two peaks were metformin and ranitidine, respectively.

Moreover, the results of the purity index in *Figure 2* showed that the two peaks having high purity index (0.999). Therefore, the method has good specificity.

Table 2. The results of specificity (n = 6)

S.No	Response (mAU.s)		Respone rate (%)	Response (mAU.s)		Respone rate (%)
	Blank sample	LLOQ (Met 0.1 µg/ml)	Blank/Met (< 20 %)	Blank sample	LLOQ (Met 0.1 µg/ml)	Blank/Ran (< 5 %)
1	0	11.6	0	0	57.6	0
2	0	10.4	0	0	53.6	0
3	0	10.7	0	0	54.7	0
4	0	10.0	0	0	53.8	0
5	0	11.8	0	0	54.8	0
6	0	11.0	0	0	54.4	0

Besides, the signals of interfering components can be acceptable where the responses in the blank samples are less than 20% (for the analyte) and 5% (for the internal standard) of those in the LLOQ samples (*Table 2*). Thus, the specificity of the method is in accordance with US-FDA 2018 and EMA 2011.

3.2.3. Linear range

Analyze standard samples of metformin in plasma with the range of concentration 0.1 – 4.0 µg/ml, each concentration was analyzed 5 times on five consecutive days. The linear relationships between the concentration of metformin in plasma and the peak area ratio of metformin/ranitidine are presented in *Table 3*.

Table 3. Results of linear range (n = 5)

Sample	Concen tration (µg/ml)	Accuracy (%)				
		CC1	CC2	CC3	CC4	CC5
S1	0.1	81.72	96.11	82.59	93.49	115.30
S2	0.2	88.62	98.46	89.46	109.50	103.21
S3	0.5	100.59	104.03	99.30	101.33	101.55
S4	1.0	102.55	99.79	95.91	95.38	94.99
S5	2.0	105.34	102.18	106.52	102.69	103.31

S6	3.0	95.25	95.88	100.72	98.69	96.87
S7	4.0	101.21	101.74	98.26	100.31	101.20
Linear regression (Y= a + bX) b = 1.4685 r^2 = 0.9974		a = 0.0799 b = 1.4661 r^2 = 0.9984	a = 0.059 b =1.5181 r^2 = 0.9982	a = 0.0449 b = 1.5546 r^2 = 0.9995	a = 0.0156 b = 1.5312 r^2 = 0.9987	a = -0.0283

(Y: the peak area ratio of metformin/ranitidine: X: the concentration of metformin in plasma)

In the concentration range of 0.1 – 4.0 µg/ml, there are linear relationships between the concentration of metformin and the peak area ratio of standard/internal standard with the correlation of coefficient (r) of all five calibration curves ≥ 0.98. Metformin concentrations determined from the calibration curve compared with the actual concentration were within the allowable range (80 - 120% for the lowest concentration and 85 - 115% for other concentrations).

3.2.4. Lower Limit of Quantification (LLOQ)

LLOQ was determined by gradually lowering the concentration of metformin in human plasma (0.2 µg/ml, 0.1 µg/ml, and 0.05 µg/ml). At each concentration, analyzing 6 independent samples (n = 6). Calculate metformin concentrations in the standard samples based on a daily calibration curve, then determine LLOQ value based on the accuracy and the peak response ratio of metformin in standard samples compared to blank samples. At the concentration of 0.1 µg/ml, metformin meets the LLOQ requirement of the analytical method in biological fluids. The results were shown in *Table 4.*

Table 4. Results of lower limit of quantification (n = 6)

S.No	Blank sample	Standard sample (0.1 µg/ml)			
	Met (mAU.s)	Met (mAu.s)	IS (mAu.s)	Concentration found (µg/ml)	Accuracy (%)
1	0	11.6	57.6	0.0969	96.92
2	0	10.4	53.6	0.0919	91.90
3	0	10.7	54.7	0.0930	92.98
4	0	10.0	53.8	0.0863	86.34
5	0	11.8	54.8	0.1064	106.43
6	0	11.0	54.4	0.0975	97.48
Mean	0	10.9	54.8	0.0953	95.34
	SD			0.0068	6.76
	RSD (%)			7.09%	

The accuracy compared with the actual concentration was from 86.34 to 106.43% (in the range from 80 to 120%), the repeatability of the method after 6 analyses met the guideline on validation of analytical methods of US-FDA and EMA with the relative standard deviation less than 20% (7.09%). Thus, the human plasma sample with metformin 0.1 µg/ml met the LLOQ requirement of the bioanalytical method.

3.2.5. Accuracy, precision

The intra-day accuracy and intra-day precision were evaluated by analyzing quality control (QC) samples at three levels of concentration: Lower Quality Control (LQC = 0.3 µg/ml), Medium Quality Control (MQC = 2.0 µg/ml), High Quality Control (HQC = 3.0 µg/ml). At each concentration, analyzing 6 independent samples (n = 6), the results were shown in *Table 5*.

Table 5. The results intra-day accuracy and precision of the CE method (n = 6)

S.No	LQC (0.3 µg/ml)		MQC (2.0 µg/ml)		HQC (3.0 µg/ml)	
	Concentration found (µg/ml)	Accuracy (%)	Concentration found (µg/ml)	Accuracy (%)	Concentration found (µg/ml)	Accuracy (%)
1	0.2983	99.45	2.0303	101.51	2.9248	97.49
2	0.3058	101.94	2.1510	107.55	3.0713	102.38
3	0.2839	94.64	2.0930	104.65	3.2412	108.04
4	0.3171	105.69	2.1292	106.46	3.2769	109.23
5	0.2949	98.32	2.1072	105.36	3.1352	104.51
6	0.2893	96.43	2.1232	106.16	3.1339	104.46
Mean	0.2982	99.41	2.1056	105.28	3.1305	104.35
RSD%	3.99%		1.99%		4.03%	

The inter-day accuracy and inter-day precision were evaluated by analyzing quality control (QC) samples at three levels: LQC (0.3 µg/ml), MQC (2.0 µg/ml), HQC (3.0 µg/ml). This was done by analyzing a set of samples (n = 6) at each level on three consecutive days. Samples were processed and analyzed under the optimum separation conditions. Metformin concentrations in samples were determined based on the calibration curve in the same day. The results were shown in *Table 6*.

Table 6. The results inter-day accuracy and precision of the CE method (n = 6)

Date	S.No	LQC (0.3 µg/ml)		MQC (2.0 µg/ml)		HQC (3.0 µg/ml)	
		Concentration found (µg/ml)	Accuracy (%)	Concentration found (µg/ml)	Accuracy (%)	Concentration found (µg/ml)	Accuracy (%)
I	1	0.2983	99.45	2.0303	101.51	2.9248	97.49
	2	0.3058	101.94	2.1510	107.55	3.0713	102.38
	3	0.2839	94.64	2.0930	104.65	3.2412	108.04
	4	0.3171	105.69	2.1292	106.46	3.2769	109.23
	5	0.2949	98.32	2.1072	105.36	3.1352	104.51
	6	0.2893	96.43	2.1232	106.16	3.1339	104.46
II	1	0.2633	87.76	1.9769	98.85	2.9973	99.91
	2	0.2629	87.64	2.1234	106.17	3.2083	106.94
	3	0.2717	90.57	2.0571	102.86	3.2376	107.92
	4	0.2685	89.51	2.1517	107.58	3.1711	105.70
	5	0.2716	90.54	2.0754	103.77	3.1045	103.48
	6	0.2635	87.84	2.1381	106.91	3.1259	104.20
III	1	0.2652	88.40	2.1209	106.04	2.8935	96.45
	2	0.2679	89.32	2.1727	108.63	3.2282	107.61
	3	0.2670	89.00	2.2645	113.23	3.2322	107.74
	4	0.2744	91.48	2.1877	109.39	3.2134	107.11
	5	0.2667	88.90	2.1380	106.90	3.1221	104.07
	6	0.2612	87.06	2.2122	110.61	3.0840	102.80
Mean		0.2774	92.47	2.1251	106.26	3.1334	104.45
RSD%		6.05%		3.11%		3.48%	

The results showed that the method has the intra-day and inter-day accuracy were 99.41 – 105.28% and 92.47 – 106.26%, respectively, within the permissible range (85 – 115%). The intra-day and inter-day precision were 1.99 – 4.03% and 3.11 – 6.05%, respectively, within the acceptance range (less than 15%). Thus, the developed method met the requirements of the accuracy and precision of the bioanalytical method.

3.2.6. Recovery (The extraction efficiency)

The QC samples at three levels LQC (0.3 µg/ml), MQC (2.0 µg/ml), HQC (3.0 µg/ml) were analyzed by the developed method. In parallel, the standard samples in water with the same concentration (without extraction) were also analyzed by the CE method. The recovery was determined by comparing the peak areas of metformin in extracted samples to the peak areas of the same amounts of metformin directly injected into the instrument, without extraction. The results were shown in *Table 7* and *Table 8*.

Table 7. The results of recovery of metformin (n = 6)

S.No	LQC (0.3 µg/ml)			MQC (2.0 µg/ml)			HQC (3.0 µg/ml)		
	Met/P	Met/W	Recovery (%)	Met/P	Met/W	Recovery (%)	Met/P	Met/W	Recovery (%)
1	29.9	40.6	73.6	178.8	259.0	69.0	296.9	393.3	75.5
2	29.9	41.6	71.9	191.8	256.3	74.8	313.4	389.8	80.4
3	28.2	40.5	69.6	193.6	247.3	78.3	310.3	391.8	79.2
4	31.5	41.3	76.3	188.3	243.0	77.5	305.9	402.5	76.0
5	29.6	40.1	73.8	193.0	237.1	81.4	305.4	397.8	76.8
6	29.1	40.4	72.0	193.8	258.0	75.1	307.6	400.8	76.7
Mean	29.7	40.8	72.9	189.9	250.1	75.9	306.6	396.0	77.4
RSD%	3.67	1.41	3.08	3.05	3.61	5.50	1.83	1.30	2.50

Table 8. The results of recovery of ranitidine (n = 18)

S.No	1	2	3	4	5	6	7	8	9	Mean (%)	RSD (%)
Ran/P	60.2	58.9	59.3	60.1	60.2	60.2	58.9	59.7	61.9		
Ran/W	70.7	68.3	69.2	68.9	68.5	68.2	72.8	69.5	74.2		
S.No	10	11	12	13	14	15	16	17	18		
Ran/P	59.2	61.3	61.1	68.3	66.5	64.5	62.9	65.6	66.1	86.2	4.74
Ran/W	74.8	68.7	74.3	73.7	70.5	79.4	75.1	75.4	72.5		

P: Plasma, W: Water

The results showed the mean recovery of ranitidine (internal standard) was 86.2%, the mean recoveries of metformin at three levels LQC, MQC, and HQC were 72.9%, 75.9%, and 77.4%, respectively. Recovery of metformin and the internal standard had good repeatability (RSD for the analyte less than 10%, RSD for the internal standard less than 5%).

Therefore, the sample preparation procedure developed is suitable for the extraction of metformin in human plasma.

3.2.7. Stability

The short-term and long-term stabilities of the stock solution of the analyte (metformin 100 µg/ml), the stock solution of the internal standard (ranitidine 100 µg/ml), the working solution of the internal standard (ranitidine 40 µg/ml) were evaluated by analyzing under the optimum separation conditions. The results were shown in *Table 9*, *Table 10,* and *Table 11*.

Table 9. The results of short-term stability of metformin 100 µg/ml and ranitidine 100 µg/ml (n = 3)

S.No	Metformin 100 µg/ml (mAU.s)		Ranitidin 100 µg/ml (mAU.s)	
	Initial	After 5 hours	Initial	After 5 hours
1	682.4	680.5	156.9	155.5
2	680.2	679.2	158.1	157.2
3	683.3	679.1	160.3	157.8
Mean	681.9	679.6	158.4	156.8
RSD%	0.23%	0.11%	1.09%	0.76%
Deviation %	-0.35%		-1.31%	

Table 10. The results of long-term stability of metformin 100 µg/ml and ranitidine 100 µg/ml (n = 3)

S.No	Metformin 100 µg/ml (mAU.s)		Ranitidin 100 µg/ml (mAU.s)	
	Initial	After 30 days	Initial	After 30 days
1	682.4	683.7	156.9	161.6
2	680.2	684.2	158.1	160.1
3	683.3	680.8	160.3	157.2
Mean	681.9	682.9	158.4	159.6
RSD%	0.23%	0.27%	1.09%	1.40%
Deviation %	0.14%		0.76%	

Table 11. The results of short-term stability of ranitidine 40 µg/ml (n = 3)

S.No	Ranitidin 40 µg/ml (mAU.s)	
	Initial	After 5 hours
1	67.7	65.9
2	65.2	65.3
3	65.8	64.9
Mean	66.2	65.4
RSD%	1.97%	0.77%
Deviation %	-1.31%	

The results showed that concentrations of the stock solutions of the analyte, the stock solution of the internal standard, the working solutions of the internal standard after a short-term storage at room temperature ($25^0C \pm 2^0C$, 75% ± 5% RH) in 5 hours and after a long-term storage at $2\text{-}8^0C$ for 30 days has a deviation less than 2% of those of the freshly prepared solutions.

Next, the stabilities of the analyte in the matrix were evaluated under different conditions as follows: at the room temperature for 5 hours; in the auto-sampler for 24 hours; in the freezer at -20^0C for 30 days; and after three freeze-thaw cycles. This was done by analyzing a set of quality control (QC) samples (n = 6) at two levels LQC (0.3 µg/ml), HQC (3.0 µg/ml). Samples were processed and analyzed under the optimum separation conditions. The metformin concentrations in the samples were determined based on the calibration curve conducted each day. The results were shown in *Table 12*.

Table 12. Stability results of metformin in matrix (n = 6)

Storage conditions	Sample	At the beginning (Mean concentration, μg/ml, n = 6)	After storage (Mean concentration, μg/ml, n = 6)	RSD (%)	Deviation (%)
After thawing at room temperature for 5 hours	LQC	0.3286	0.3141	1.02	-4.41
	HQC	2.8634	2.7115	1.95	-5.30
In the auto-sampler for 24 hours	LQC	0.3286	0.3035	3.70	-7.64
	HQC	2.8634	2.7217	4.64	-4.95
In the freezer for 30 days	LQC	0.3286	0.2927	3.77	-10.93
	HQC	2.8634	2.8670	3.27	0.12
After three freeze-thaw cycles	LQC	0.3286	0.2836	1.46	-13.69
	HQC	2.8634	2.8960	3.90	1.14

The results showed that concentrations of metformin in the samples after different storage conditions (at room temperature after a short-term storage (for 5 hours); in the auto-sampler for 24 hours; in the freezer after a long-term storage (for 30 days); after three freeze-thaw cycles) had a deviation less than 15% as compared with those in the initial samples. Besides, RSD at each level is less than 15%. Therefore, samples can be stable at the above storage conditions before analysis.

4. DISCUSSION

In the world, there have been a number of studies on determining metformin in biological fluids by different methods, of which the most commonly used methods are high-performance liquid chromatography (HPLC) [6, 10, 11] and capillary electrophoresis (CE) [12, 13, 15]. In Vietnam, the methods for determining metformin in biological samples by high-performance liquid chromatography have also been reported [1, 2, 5]. In general, the HPLC method usually gives good separation results, high accuracy and sensitivity, but has the high cost of sample analysis and is solvent-consuming.

In the past few years, capillary electrophoresis (CE) has emerged to be an important tool in the analysis, due to its separation efficiency, low amount of sample and reagent consumption, speed of analysis, and applications to a wider selection of analytes. In 1998, this method was applied by J. Z. Song and colleagues to determine metformin in human plasma, using phenformin hydrochloride as an internal standard. Although the ion-pair extraction method used for determining metformin was quite complicated, the recovery was still not good (the recoveries of metformin at three levels LQC, MQC, HQC were 80.24 %, 67.44%, and 58.97%, respectively) [13].

Inheriting and continuing to improve the method of determination of metformin in human plasma by capillary electrophoresis, our study prepared samples by protein precipitation using acetonitrile as the precipitation-induced agent. That was quite simple, time-saving and the recovery was good (the recoveries of metformin at three levels LQC, MQC, HQC were 72.9%, 75.9%, and 77.4%, respectively). Furthermore, our study has improved lower limit of quantitation (LLOQ = 0.1 μg/ml < LLOQ = 0.25 μg/ml) and RSD% is also smaller (7.09% < 15.6%) compared with Song's study.

In 2008, Phan Quynh Lan and colleagues applied the HPLC method for determining metformin in human plasma [5]. Compared with that study, our study chose ranitidine hydrochloride as an internal standard. This was done to reduce the errors during sample preparation. Besides, our method has a shorter analysis time (sample run time by CE is about 8 minutes compared to 12 minutes by HPLC), a large number of theoretical plates (CE: 10^5 – 10^6; HPLC: 10^3 - $4x10^4$) leads to excellent separation efficiency, narrower peaks, and better resolution. The analytes moved evenly in the capillary under the influence of the electrophoretic mobility create moving bands instead of parabolic moving regions like in HPLC. However, LLOQ of our CE method is higher than that of the HPLC method. For CE, the sensitivity is not as good as HPLC because the amount of sample injected is smaller than that of the HPLC. However, the LLOQ in our method still met the requirements of US-FDA 2018 and EMA 2011 to determine metformin in plasma.

The LLOQ was determined by gradually lowering the concentration of metformin and quantified using the CE method developed. Finally, the study

determined that LLOQ was 0.1 µg/ml. According to a clinical study about the pharmacokinetics of metformin, the maximum plasma concentration of metformin (Cmax) was determined approximately 2 µg/ml after administration of metformin at 1000 mg for 2 hours [8]. In accordance with the requirements of FDA - 2018 and EMA 2011, LLOQ ≤ 1/20 Cmax [9, 14]. Thus, LLOQ is 0.1 µg/ml that our study has developed meets the requirements. The method was shown to be applicable for research involving the determination of metformin in clinical studies.

5. CONCLUSIONS

A rapid and sensitive capillary electrophoresis method has been developed and validated for the determination of metformin in human plasma with ranitidine hydrochloride as an internal standard. A 100 mM phosphate buffer solution (pH = 4) was found to be suitable for separation. An uncoated fused-silica capillary column of a total length of 40 cm (31.5 cm effective length) was used for separation. All the analytes were completely separated within 3 min at an applied voltage of 15 kV, sample injection mode 50 mbar in 7 seconds, and the detection was performed at 232 nm. Validation of the CE method for the quantification of metformin in plasma showed that the method had systematic suitability, high sensitivity, suitable linear range, high extraction recovery, low limit of quantification, precision, accuracy, and stability in accordance with US-FDA 2018 and EMA 2011. This analytical method might be applicable to the therapeutic drug monitoring of metformin and it might be used to investigate the relationship of the concentration of metformin in plasma and the therapeutic effectiveness in diabetic type 2 patients.

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