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New HPLC method for determination of cystatin C biomarker in human blood

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ABSTRACT

A rapid and sensitive method for determination of cystatin C (CC) protein in human blood by means of high performance liquid chromatography (HPLC) was developed and validated. Estimated glomerular filtration rate (eGFR) has been calculated for the patients with chronic kidney disease (CKD). Ion pair liquid chromatography technique was utilized to separate CC along with UV detection. Calibration curve with excellent correlation (r^2 = 0.99) over the range from 0.75 to 20.50 mg/L of CC was accomplished. Limits of detection and quantification were 0.375 and 0.75 mg/L of CC, respectively. The recoveries were in the range of 93.6-102.3%. Intra-assay and inter-assay variabilities were 8.2 and 6.14%, respectively.

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1. Introduction

Excretion of waste is one of the essential functions of the kidney. Any interruption of the kidney results in renal failure and it can be acute or chronic [1]. In case of renal failure different types of substances (mainly proteins) are excreted such as: creatinine, micro albumin (defined as urinary albumin excretion 30-300 mg/day, or 20-200 µg/min), CC, hence, their levels expand as the glomerular filtration rate decreases, these proteins are called kidney failure biomarkers [2]. These biomarkers interact in different manners to reflect the situation of the kidney, and they are classified as exogenous markers, such as: ^{51}Cr-EDTA, inulin, (^{125}I), iotholamate (^{99m}Tc) TPA and endogenous markers such as: microalbuminuria, creatinine, cystatins, *etc.* [3-5]. After a careful review of each marker's properties, it was noticed that CC is an ideal and reliable marker of GFR, and so of renal functions. CC is a cysteine proteinases inhibitor. Large number of pathological processes is controlled by the balance between proteinases and their inhibitors, where an increase of these proteinases has been observed in case of inflammation. The concentration of CC protein is considered as an overall index of renal function. In case of GFR reduction, CC leaks out from the kidney. CC amount in the blood increases by two folds at the early stages of kidney failure [6-11].

Different immunoassay methods have been reported for determination of CC: Particle enhanced nephelometric immuneassay (PENIA), particle enhanced turbidimetric method (PET) and enzyme-amplified single radial immunediffusion [12-14]. Due to the interferences caused by antigens-antibodies cross reactions which might lead to false positive results, long analysis time, in addition, to the high coefficient of variation obtained by the mentioned methods, the development of a new, specific, fast and precise method is deemed necessary. HPLC-MS analytical method is in use for the determination of CC only in raw material but has not been applied to biological fluids [15]. Another HPLC method has been developed and validated based on isotope dilution liquid chromatography principles with electrospray ionization detection, however, no real samples were introduced as well [16].

To the best of our knowledge, there is no chromatographic method of analysis has been developed for the sake of analyzing CC in human blood, however, a method has been developed and validated in our previous work to analyze CC in human urine [17,18]. The present work introduces a new, fast, economic HPLC method which can separate, detect and quantify CC by simple preparation steps, it also conquers the problems of the previous immunoassay techniques. The obtained values of CC will be incorporated to calculate eGFR

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values of the CKD patients. Based on the calculated eGFR values the patients could be classified into different kidney failure stages.

2. Experimental

2.1. Chemicals

CC protein (>96%) was purchased from BBI Solutions (UK), HPLC grade acetonitrile, methanol, 1-hexane sulfonic acid sodium salt, trifluoroacetic acid (TFA) and acetone were purchased from Merck (Germany).

2.2. Blood samples collection and processing

17 males and females (range 8-58 years old) of healthy volunteers were recruited. 27 Males and females patients (range 13-85 years old) with known history of renal failure disease were recruited as well from hospital (Extra samples from the medical laboratory). Implied consent has been obtained. Blood samples were collected from all groups into heparinized tubes and were kept cooled in an ice box.

2.3. Sample preparation

Preparation steps were carried out in a cold room. Blood samples were collected in heparinized tubes and centrifuged at (4400 × g at 4 °C) for 5 minutes. The supernatant plasma was separated gently by a dropper to another tube. To precipitate CC; 2 mL of plasma solution was withdrawn in a test tube and about 8 mL of the acetone was added, then the resulting solution was mixed thoroughly by shaker for 20 minutes. After that, centrifugation at (4400 × g at 4 °C) for 30 minutes took place and the resulting solid precipitate was dissolved in 4 mL 0.05% TFA (*v*:*v*). Finally, the solution was centrifuged at (4400 × g at 4 °C) for 30 minutes and the supernatant was filtered through 0.45 µm teflon filter and injected into the HPLC system.

2.4. Chromatographic conditions

Dionex HPLC system (Dionex, Germany) with a degasser, low pressure gradient pump, column oven, an autosampler, a UV detector was used. Data acquisition was performed with Chromeleon 7.2 SR2 software. Another HPLC instrument was used to perform the inter-assay (ruggedness) test; (LC2010 HPLC system, Shimadzu, Japan), Data acquisition was performed with LC Solution 1.25 SP2 Software. A reversed phase Ace C8 (150 × 4.6 mm i.d., 5 µm) column (supported with a guard holder contains Ace 5 C8 100A guard cartridge) was placed in the column oven at 25 °C. Mobile phase A of 0.01 M 1-hexane sulfonic acid sodium salt plus 0.05% TFA, pH = 2.4 filtered through 0.45 µm teflon filter and mobile phase B (acetonitrile:methanol:mobile phase A) (300:300:225, *v*:*v*:*v*), pH = 2.5 filtered through 0.45 µm teflon filter, were pumped at a flow rate of 1.0 mL/min and used for the elution of CC utilizing a gradient program. Before each run, the column was equilibrated with 65% of mobile phase B for 3 minutes. The gradient was increased gradually to 80% in 3 minutes and then to 100% in 5 minutes lasted 2 minutes to complete the separation. Then, the gradient was decreased to initial conditions 65% in 0.5 minute. At 14 minutes, the HPLC system is ready for the next injection. The injection volume was 100 µL. The eluent was monitored at 224 nm.

2.5. Assay validation

Validation tests were conducted according to the ICH Guideline Q2 (R1) [19] and Bioanalytical Method Validation Guidance [20].

2.5.1. Linearity

To build up the calibration curve, six points were constructed from CC stock solution (0.2 mg/mL of CC in healthy blood plasma). Same sample preparation procedure was applied.

2.5.2. Limits of detection (LOD) and quantification (LOQ)

From the calibration curve and according to the visual evaluation method, the LOD is defined as the lowest concentration that can be detected but not necessarily quantified and the LOQ is the lowest concentration that can be determined with acceptable accuracy and precision.

2.5.3. Accuracy and precision

Stock solution of CC was prepared in healthy plasma (0.2 mg/L of CC), after that, appropriate dilutions were carried out using healthy plasma to construct the quality control samples with the following concentrations: 10.00, 5.00 and 2.50 mg/L of CC, high, medium and low, respectively. In order to mimic the exact environment of the real sample; the final amount of the plasma was kept constant in all levels (0.5 mg/L of CC) during the dilution steps. Further dilutions have been performed from the lowest prepared concentration (2.50 mg/L of CC) to estimate LOD and LOQ. Quality control (QC) samples were stated toward the beginning and the end of assay work to determine the accuracy and the precision. RSD's were determined from 6 preparations (inter-day/ruggedness) and from 10 independent assays from one experimental day (intra-day).

2.5.4. Specificity

To check the purity of the peak attributed to CC in real sample, peak purity index and similarity factors values were determined using a photo diode array (PDA) detector.

2.5.5. Selectivity

Different over the counter drugs (OTC) were tested (in our previous study) to check for any interference with the peak due to CC [17].

2.5.6. Stability of analytical solutions

Two stability study approaches have been triggered; one on the final analytical solutions and the other one is on the plasma solutions.

2.5.6.1. Stability of final analytical solutions (all preparation steps were applied)

These solutions refer to those obtained after applying all the preparation steps. Stability solutions were prepared by storing prepared analytical samples for 2 days at room temperature, in the refrigerator, and in the freezer (25 °C, 2 to 8 °C and -10 to -20 °C), respectively. The concentrations were calculated from the calibration curve and compared with the actual values.

2.5.6.2. Stability of plasma solutions

Stability solutions were prepared by storing plasma samples for 8 days at room temperature, in the refrigerator, and in the freezer (25 °C, 2 to 8 °C, and -10 to -20 °C), respectively. Then the sample preparation procedure was applied. The concentrations were calculated from the calibration curve and compared with the actual values.

Table 1. Accuracy and precision results.

Concentration (mg/L)	QC Sample	Accuracy CC (%)	%RSD
10.50	MAX	93.6±15	8.4±15
5.50	MEDIUM	98.9±15	6.9±15
3.00	LOW	96.4±15	6.4±15
0.75	LLOQ	99.3±20	12.5±20

Table 2. Within-lab variation/ruggedness results.

Prep	Day 1 CC (mg/L)	Day 2 CC (mg/L)	Average	%RSD
1	0.96	0.76	0.86	8.3±15
2	1.13	1.04	1.08	3.0±15
3	1.42	1.57	1.50	3.6±15
4	1.20	1.76	1.48	13.4±15
5	1.37	1.35	1.36	0.5±15
6	0.72	0.57	0.65	8.0±15
Average			1.15	6.14±15

3. Results

3.1. Absorbance spectra of CC

CC has absorption maximum at around 224 nm.

3.2. Specificity

Peak purity index and the similarity of the 3 point peak purity values have shown that the detected peak is only attributed to CC (0.999737 and 0.999378, respectively). Moreover, no impurities have been detected which might interfere with the peak due to CC.

3.3. Selectivity

No interferences were observed between the peaks due to the OTC drugs and the peak due to CC (Figure 1).

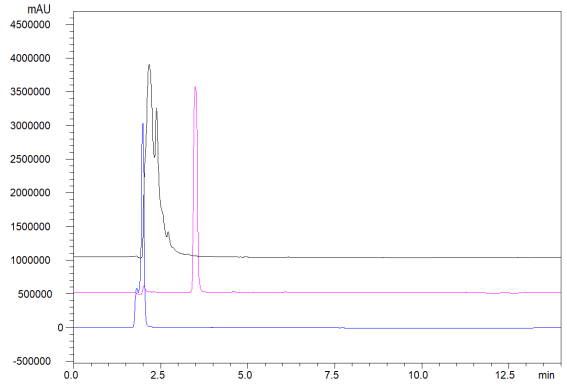

Figure 1. Chromatograms of OTC drugs (Nicotine, Aspirin and Ascorbic Acid). Nicotine (black); aspirin (pink) and ascorbic acid (blue) [17].

3.4. System precision (Intra assay)

Ten replicate injections of CC solution have been performed to check the suitability of the HPLC instrument. The obtained RSD% ascertained that the HPLC system was working fine (8.2%). Additional five replicate injections of CC standard solution have been performed to check the suitability of the other HPLC instrument (LC2010) and the obtained RSD% was also within the acceptable limits (5.6%).

3.5. Linearity

The peak areas of CC were linear with respect to the concentrations over the range 0.75-20.50 mg/L of CC. Excellent correlation was obtained r^2 = 0.9931, (y_{mAU} = 45.025 × x + 10.988). Data was analyzed using "linear regression least squares fit".

3.6. Accuracy and precision

Accuracy and precision were established by analyzing high, medium and low concentrations of QC samples throughout the standards calibration range. Intra-assay and inter-assay precisions were < 15% (8.2% and 6.14, respectively), and the mean values of the accuracy were between 93.6 and 102.3% (Figure 2, Table 1). LOQ and LOD were confirmed to be 0.75 mg/L and 0.375 mg/L of CC, respectively.

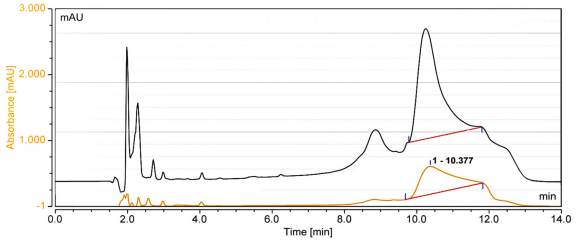

Figure 2. Chromatograms of recovery preparations 3.0 (orange) and 10.5 (black) mg/L of CC.

3.7. Intermediate precision test (Within-lab variation/Ruggedness)

Six healthy volunteers' samples (Those were tested previously; their plasma samples were kept for less than 8 days in the refrigerator) have been prepared by another analyst in an alternative day using another instrument (Shimadzu LC 2010) in another lab to validate the precision of the developed method. Acceptable %RSD has been obtained for each healthy sample (Table 2).

3.8. Stability of analytical solutions

3.8.1. Stability of final analytical solutions (all preparation steps were applied)

Stability solutions were prepared by storing prepared analytical samples for 2 days at room temperature, in the refrigerator and in the freezer (25 °C, 2 to 8 °C and, -10 to -20 °C), respectively. The concentrations were calculated from the calibration curve and compared with the actual values (Table 3).

3.8.2. Stability of plasma solutions

Stability solutions were prepared by storing plasma samples for 8 days at room temperature, in the refrigerator and in the freezer (25 °C, 2 to 8 °C, and -10 to -20 °C), respec-

Table 3. Stability of prepared solutions results.

Period	Condition	CC (%)
1 Day	Room temperature	94.7
2 Days		46.0
1 Day	Fridge (4-8 °C)	98.3
2 Days		53.8
1 Day, 1st Thaw cycle	Freezer (-10 – -20 °C)	86.4
2 Days, 2nd Thaw cycle		53.2

Table 4. Stability of plasma solutions results.

Period	Condition	CC (%)
8 Days	Room temperature	99.1
8 Days	Fridge (4-8 °C)	97.3
8 Days	Freezer (-10 - -20°C)	99.6

Table 5. Healthy samples results.

No	Gender	Age	CC (mg/L)
1	Male (Smoker)	41	0.73
2	Male	37	0.88
3	Male	33	0.48
4	Male (Smoker)	35	1.77
5	Male (Smoker)	34	1.87
6	Male	57	1.12
7	Male	57	1.71
8	Female	12	0.76
9	Female	42	0.72
10	Male	19	1.37
11	Male	20	1.19
12	Male	23	0.96
13	Male	8	1.42
14	Male	16	1.13
15	Male	43	1.08
16	Female	58	1.18
17	Male	53	0.47
Min.		8.0	0.47
Max.		58.0	1.87
Average		34.6	1.11

tively. Then the sample preparation procedure was applied. The concentrations were calculated from the calibration curve and compared with the actual values (Table 4).

3.9. Real samples/application

The concentrations of CC in healthy and pathological blood samples were measured. The obtained concentrations were in line to what have been reported in the literature by reference [5]. Mean and range are 0.96, 0.57-1.79 mg/L of CC, respectively. The obtained results by our method; mean and range: 1.11, 0.47-1.87 mg/L of CC, respectively (Figure 3, Table 5). CC concentration in the blood of the kidney failure patients has increased by 2 folds and more with respect to the reference healthy range, ($p < 0.0001$ and $r = 0.857441$) (Figure 4, Table 6).

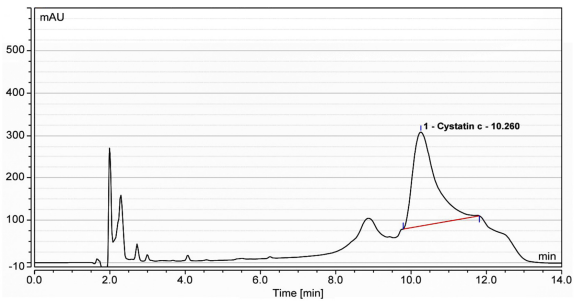

Figure 3. Chromatograms of healthy sample.

3.10. Comparative study

To compare the obtained results from the currently validated HPLC method with that generated from the available classical methods, 4 samples have been analyzed by our HPLC method and then sent to a research laboratory to be analyzed utilizing their PENIA method. A significant difference was observed between HPLC and PENIA methods, in which, the obtained means were 3.01 mg/L *vs* 6.91 mg/L of CC, respectively, ($p < 0.05$ with a good correlation $r = 0.987953$) (Table 7).

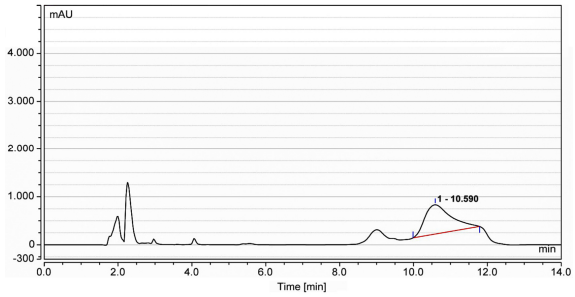

Figure 4. Chromatograms of pathological sample.

4. Discussion

4.1. Stability of final analytical solutions

The observed decay behavior could be ascribed to the effect of acidic pH value of the used solvent (0.05 % TFA) on CC [21]. In addition, the freeze/thaw cycles have a direct role in the surface-induced denaturation phenomenon which affects the proteins [22]. Interestingly, the same behavior was also noticed for the stability of CC in the urine matrix at a concentration as low as 2 mg/L of CC [17]. One-day stability for the finally prepared solution is considered fine, as long as the samples will be prepared and analyzed during the day of

Table 6. CC level in kidney failure patients and eGFR results.

No	Gender	Age	Creatinine (mg/dL)	CC (mg/L)	eGFR CC *	Stage
1	Male	25	2.89	4.39	12.56	5
2	Male	84	2.25	4.41	9.86	5
3	Male	28	6.55	5.71	8.74	5
4	Male	22	4.80	5.97	8.44	5
5	Female	50	2.59	5.81	0.28	5
6	Male before dialysis	31	2.66	3.34	17.58	4
7	Male after dialysis	31	2.66	2.00	34.71	3
8	Male	32	3.30	8.48	5.09	5
9	Female before dialysis	77	1.95	6.01	0.04	5
10	Female after dialysis	77	1.95	3.81	0.07	5
11	Female	49	3.65	4.88	0.38	5
12	Female	23	2.70	3.23	4.12	5
13	Female	51	3.16	3.40	0.54	5
14	Female	74	1.96	2.72	0.14	5
15	Female	56	2.53	2.93	0.46	5
16	Male	85	4.21	2.46	21.28	4
17	Male	45	6.51	2.74	21.64	4
18	Male	68	1.92	2.61	21.05	4
19	Male	49	5.78	2.55	23.42	4
20	Male	24	5.90	2.72	23.78	4
21	Male	44	6.83	3.37	16.55	4
22	Female	76	2.70	2.93	0.11	5
23	Female	13	4.56	5.23	4.40	5
24	Female	55	1.83	4.43	0.28	5
25	Female	47	3.44	5.16	0.41	5
26	Male	52	1.50	2.64	22.07	4
27	Female	51	1.46	3.39	0.54	5
Min		13	1.46	2.00	0.04	
Max		85	6.83	8.48	34.71	
Average		48.9	3.42	3.97	9.58	

* CKD-EPI CC equations [25]:
Female or male ≤0.8 = 133 × (Scys/0.8)$^{-0.499}$ × 0.996^{Age} [× 0.932 if female]
Female or male >0.8 = 133 × (Scys/0.8)$^{-1.328}$ × 0.996^{Age} [× 0.932 if female]

Table 7. Comparative study.

No	Assay (mg/L of CC) - HPLC Method	Assay (mg/L of CC) - PENIA Method
1	2.35	4.75
2	2.93	6.64
3	3.37	6.65
4	3.39	9.59

the preparation, however, for longer stability periods, plasma samples can be considered instead as CC showed more stability in plasma.

4.2. Stability of plasma solutions

One of the stability studies has suggested that CC is stable in plasma up to one 72 hours at room temperature [23], whereas, another study has proved the stability of CC in plasma for one week at room temperature [11]. One more stability study showed that CC is stable for 26 days at room temperature; the current stability study showed that CC is stable for 8 days in plasma at the mentioned storage conditions. This stability sounds magnificent for the therapeutic research facilities, as efforts and expenses can be both minimized, in which, more specimens can be collected and then analyzed at once.

4.3. Real sample analysis

Some of the studies have reported that the production of CC is not influenced by inflammation or any other non-renal factors [13], while the others have reported the inverse [24]. To verify or refute these hypotheses; three samples from smoker healthy volunteers have been analyzed. For one of the analyzed sample, low level of CC was recorded (sample 1, 0.73 mg/L of CC), which verifies the first hypothesis. On the other hand, the other two samples showed high levels of CC (sample 4 and 5, 1.77 and 1.87 mg/L of CC, respectively) but still within the healthy range, which concur with the second hypothesis. We can conclude that the smoking behavior has no significant effect on CC levels; however, it is worth mentioning that extra corroborative investigations with a larger sample size need to be conducted in order to get a strong conclusion. eGFR values have been established using the eGFR CC equation stated by Reference [25] for CKD patients, accordingly, the severity stage of the renal failure was stated as well. Many studies have proved the superiority of CC marker and its higher adequacy and accuracy in estimating eGFR than creatinine, nonetheless, in some cases, there might be no major contrasts in the final eGFR values [26,27]. To challenge the hypothesis that considers CC is eliminated by filtration in the dialysis process [28], pathological samples have been analyzed before and after the dialysis process and the outcomes were in accordance with that hypothesis, taking into consideration that the new concentration of CC after the dialysis process depends on its initial value, this means that the CC amount might return to fall within the healthy range (sample 6 and 7, 3.34 and 2.00 mg/L of CC, respectively) and might continue to fall in the pathological range due to its high initial concentration (samples 9 and 10, 6.01 and 3.81 mg/L of CC, respectively).

4.4. Comparative study

The higher CC values that were obtained by PENIA method could be ascribed to the interferences that immunoassays methods suffer from (whether that alter the concentration of the analyte in the sample and/or that alter the antibody binding). Despite that fact that, in the nephelometric methods the interference is reduced by dilution, but it is still observed. Furthermore, one of the obtained results using the PENIA method was out of their linear range (sample 4, 9.59 mg/L of CC), in which, their assay covers the range of 0.23-7.25 or 8.0 mg/L of CC [5,29,30]. Accentuation on using controls and calibrators that do not differ from patients' samples ought to be considered in the immunoassays methods; this necessity is

a problematic prerequisite of such techniques, otherwise, unreliable results are highly expected [31]. The controls and/or the calibrators of PENIA method might be incompatible with the submitted samples (due to different subjects' zones), so this could be another reason behind the overestimated results we received. On the contrary, such imperfection is not expected in the HPLC methods as long as the method is selective and specific. Another factor that might have played a role to a certain extent in the results discrepancies is the difference between the adopted healthy ranges in both methods which are as follows 0.5 to 1.00 mg/L of CC for PENIA and 0.47-1.87 mg/L of CC for HPLC. As a matter of fact, all the results of immunoassays methods always need to be verified by another independent method such as chromatography [32].

5. Conclusions

New HPLC analytical method has been developed and validated to be utilized for healthy and unhealthy population. Particularly, it can be utilized as a diagnostic tool for the susceptible kidney failure patients in light of the obtained CC amounts in their blood plasma; therefore, corrective action can be in place at an early stage. Selectivity and specificity tests allow analyzing samples from different population of different zones and regions without being worried about getting unreliable results. The application of the developed HPLC method can be extended to quantify CC in other biological matrices (saliva, tears, breast milk… *etc*.). It is worth mentioning, that the assessment of pathological situation based on the obtained CC levels in the urine sample is much clearer than the analogous in the blood sample, which is ascribed to the dramatic increase of CC levels in the urine in case of kidney dysfunction (200 folds and more), whereas the increase in the blood starts from 2 folds. Accordingly, extra care must be considered preceding the assessment of results obtained from blood sample.

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