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RESEARCH ARTICLE

RP–HPLC Developed Method for Uric Acid Estimation in Human Serum

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ABSTRACT:
A sensitive, efficient, reproducible and rapid method for the specific determination of uric acid as pure and in human serum has been developed using reverse phase high performance liquid chromatographic (RP - HPLC). This method involves separation of uric acid on reverse phase HPLC Shimadzu LC–20 A, Japan and Phenomenex C18 column (250 × 4.6 mm, 5µm). The elution was done using a mobile phase consisting of sodium acetate buffer: acetonitrile (ACN) as the ratio of (95:05 v/v with pH adjusted at 4.0 using acetic acid). The separation was monitored for 10 min. at 285 nm using a UV-visible detector and 1.2 mL/min flow rate. The optimum conditions such as the composition of the mobile phase, flow rate, wavelength and pH were studied. The obtained results revealed that the value of R^2 is (0.9994), the detection limit (0.01), the quantitative limit (0.033), the linear ranges (0.05 – 30) µg / mL. Quantitative recoveries of pure uric acid and spiked serum samples were between 98.75 – 101.20%. Compared to the other methods, the current method is rapid, simple and economical for the determination of uric acid as pure and in human serum.

KEYWORDS: RP-HPLC, Sensitive, Developed, spiked**.**

INTRODUCTION:
The dietary life improvement through the economic development causes high protein over nutrient for the peoples so that the uric acid is increasing in a blood serum by a physiological reaction. Such physiological increase of uric acid is due to the over partaking of high protein foodstuffs and the uric acid is also influenced by an under exercise and a heavy stress [1]. Uric acid (UA) has long been considered an inert end product of purine catabolism in mammals. Normal levels of uric acid in the blood of men that are in the range of 3.4 to 7.0 mg/dL, while the woman is in the range from 2.4 to 5.7 mg/dL. It is not only an important indicator of physiological state, but also the cause of some diseases. For example, high levels of UA in the blood can cause hyperuricemia, which has emerged as a major health problem in industrialized nations [2,3]. Gout is a common disease due to the abnormal level of uric acid in the human body to cause the existence of crystallites which inhibit the human joints [4].

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It is also known as a type of arthritis or a uric acid disturbance in the human body which give a painful effect to the human joint bones. Besides that, kidney stone may exist through this disease which may cause renal failure [5]. The crystallites are uneasy to be dissolved in the human blood and they may cause intense pain and swelling of the human joints besides slowing down the body movements [6]. Based on the medical research perspective, uric acid concentration is a 'fingerprinting' of the risk of this disease to happen [7]. Growing demand for accuracy and rapid determination of analytes in human physiological fluids has always been observed. It is mainly focused on the devices that allow one to make simple, reliable and cheaper analysis performed close to the patients [8]. Therefore, its accurate determination in a urine is very important to make a diagnosis of the first or second symptom of a gout. Brown's reduction method and an enzymatic method by urease are widely used for the clinical determination of uric acid. But these methods have a high detection limit and are significantly tedious to process the procedure. Thus, rapid and simple methods have been tried to obtain an accurate analytical result of the uric acid in real samples such as a urine [9]. There are many standard analytical methods that have been reported in the literatures for uric acid determination. The levels of uric acid in different biological matrices such as urine and serum have been determined by

numerous standard analytical methods such as Spectrophotometric [10-14], Reversed-phase liquid chromatography [15-20], Potentiometric [21,22], Amperometric [23], Voltammetric [24] and Flow injection analysis system [25,26]. In this study, we have developed a simple, accurate method for uric acid determination in human serum. Because analytical methods must be validated before use, the proposed HPLC-UV detection method was validated in accordance with the International Conference on Harmonization (ICH). The presented method is useful for the analysis of samples where the classical method does not give reliable results.

MATERIALS AND METHODS:

Chemicals and reagents:

Uric acid (UA) standard as a pure powder was from. Methanol and Acetonitrile (HPLC-grade) were from BDH. Sodium hydrogen, sodium acetate, sodium borate, acetic acid, boric acid and phosphoric acid as buffer solutions were from BDH. Freshly prepared deionized water was used.

Instrumentation and chromatographic conditions:

HPLC (Shimadzu LC–20 A, Japan), Sartorius balance (Germany), Ultrasonic bath (Karl Kolb, Germany), Shaking water bath (Taiwan) and oven (Memmert, Germany) were used through this study. Uric acid was Phenomenex C-18 (250 × 4.6 mm I.D and 5 µm particle size). Analysis was performed at room temperature (~25 ^{o}C) and the total run time was 10 min, under reversed phase partition conditions. The mobile phase was 0.1 M acetate buffer and acetonitrile in the ratio of (95:05 v/v) adjusted at pH 4.0 using acetic acid. The flow rate was 1.2mL/min. Samples were injected using an injector with 10µL loops and detection was carried out at 285 nm. Before analysis mobile phase were degassed by the use of a sonicator (Ultrasonic Cleaner, Power Sonic 420) and filtered through a 0.45µm nylon filter. The identity of the compound was established by comparing the retention time of compound in the sample solution with those in the standard solution. Chromatography was performed in column temperature maintained at 25±5°C. The UV spectrum of uric acid selecting the working wavelength of detection was taken using a Jasco V-650 Japan double beam UV-Visible spectrophotometer with 10 mm path length quartz cells was used for the analytical purpose.

Preparation of solutions:

Standard stock solution:

Standard stock solution containing 1000µg/mL of the uric acid was prepared in 0.2 N sodium hydroxide using uric acid reference standard. Pipette out 5 ml of solution from 1000µg/mL stock solution and transfer into 50ml volumetric flask and diluted up to the mark with mobile phase to give a working standard solution having a concentration of 100µg/mL.

Diluent:

By using the stock solution of 100µg/mL, subsequent dilution was carried out by withdrawing different aliquots (0.005–3.0mL) from standard solution were transferred into a series of 10 ml calibrated volumetric flasks and all were made up to the mark with mobile phase in order to prepare working standard solutions of different concentrations (0.05–30µg/mL).

Blood sampling:

To determine the serum concentration of uric acid, 3-5 mL of whole blood was drawn from each volunteer. To obtain serum, the blood samples were then centrifuged at 4000 rpm for 15 min. at room temperature, serum was separated just after sample collection and was frozen store for subsequent assessment.

RESULTS AND DISCUSSION:

Estimation of detection wavelength:

A solution of uric acid in the concentration of 10µg/mL was scanned in the range of wavelength 200-400 nm. It was observed that the uric acid solution showed considerable absorbance at a wavelength of 285 nm. The absorption spectrum was found sharp and maximum at a wavelength of 285 nm. Therefore, it was selected as the wavelength for detection in HPLC analysis. The study of spectrum revealed that the uric acid solution was shown a well-defined λ_{max} at 285 nm as shown in (Fig. 1).

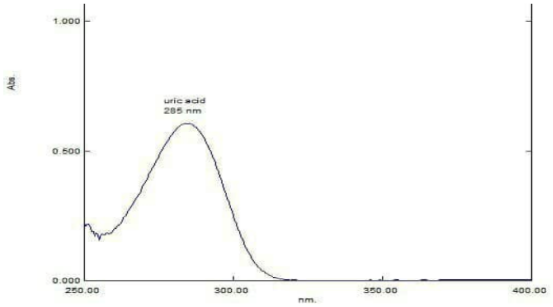

Fig: 1: UV spectra of uric acid.

Method Development:

Several tests were performed in order to get satisfactory separation-resolution of uric acid in different mobile phases with various ratios of buffers and organic phases. The ideal mobile phase was found to be a buffer and acetonitrile. This mobile phase used gave a very satisfactory and good resolution of uric acid. Increasing or decreasing the pH of the mobile phase by ± 0.2 did not show significant change in the retention time of the analyte. The retention time of uric acid on the analytical column was evaluated at a flow rate of 1.2mL/min. The

injection volume was 10µL. The retention time of standard and sample for uric acid was satisfied with good resolution in human samples. This work was focused on optimization of the conditions for the simple and rapid as well as low cost, effective analysis, including a selection of the mobile phase to obtain satisfactory results. Solvent type, solvent strength (volume fraction of organic solvent (s) in the mobile phase and pH of the buffer solution), detection wavelength, and flow rate were varied to determine the chromatographic conditions giving the best separation. The mobile phase conditions were optimized so there was no interference from solvent and excipients. The finalized predicate optimum chromatographic conditions were mentioned on below table 1. The optimized chromatogram for uric acid is shown in (Fig. 2).

Table: 1: The predicate optimum parameters for HPLC method.

Mobile phase compassion Buffer solution: ACN 95: 05	**Column type: ODS: 250mm, 4.6mm, 5µm**
	Sample temperature: ambient
Flow rate 1.2 mL/ min.	Column temperature: 25 ±5 °C
Injection volume 10 µL	Run time min 10.00
Wavelength nm 285	Retention time min 6.268

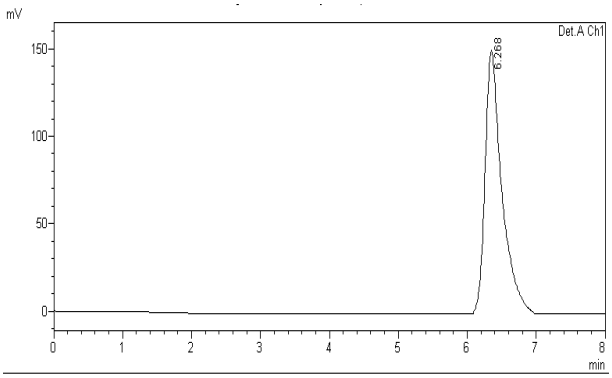

Fig: 2: Optimized chromatogram for uric acid.

Preparation of Calibration graph:

From the stock solution, subsequent dilutions were made with mobile phase to obtain the series of standard solutions have a concentration range of (0.05–30 µg/mL) of uric acid. The solutions were injected using injector loop of 10µL and chromatograms were recorded. A graph was plotted by taking a concentration on X-axis and the area under the peak on Y-axis which gave a straight line.

System Suitability Test:

The observed values of Resolution, Column efficiency, Tailing factor were depicted in table 2.

Table 2: System suitability study of uric acid.

System suitability Parameters	Results	Acceptance Criteria
Retention time	6.268	
% RSD for area of uric acid seven injections of standard solution	0.217	NMT 2.0
Talling factor for uric acid peak	1.34	NMT 2.0
Theoretical plates for the uric acid	3576	NLT 2000

Analytical method validation:

Validation of developed method was carried out as per ICH Q_2 R_1 guideline [27]. Parameters such as Linearity, Accuracy, Precision, Specificity, LOD, LOQ, Ruggedness and Robustness were taken up as tests for analytical method validation.

Linearity and Range

The proposed RP-HPLC shows good linearity in the concentration range of 0.05 to 30 µg/mL of uric acid depicted in (fig. 3). The slope and intercept value of the calibration graph were y = 173783x + 94483 (R^2 = 0.9994) for uric acid. The results are satisfactory, because there is a significant correlation between response factor and concentration of drugs within the concentration range.

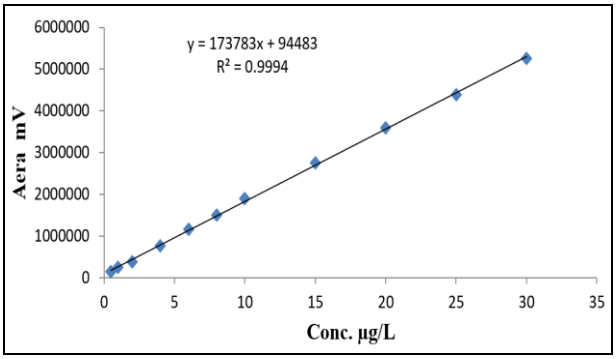

Fig: 3: Calibration graph foe uric acid in buffer: ACN (95: 05 v/v)

Precision:

The intraday precision of the developed method was evaluated by analyzing uric acid samples of different concentrations three times on the same day and %RSD was calculated. The inter day precision was evaluated by analyzing samples of different concentrations of uric acid on three different days and %RSD was calculated. Repeatability was evaluated by injecting the standard solutions of uric acid (5 µg/mL) five time on the same day and the value of %RSD were calculated. The results obtained are shown in table 2.

LOD and LOQ:

LOD and LOQ were calculated by gradual dilution of lowest concentration and as 3.0 LOD respectively. The results obtained are shown in table 2.

Accuracy:

This study was carried out to assure the closeness of the test results obtained by the analytical method to the true value [28]. For study methods, uric acid was determined at three different selected concentrations within the Beer's law limits 3, 5, 7µg/mL. The results were reported in table 3, which revealed that the suggested method for detection of uric acid interesting and quite convenient with respect to the methods and parameters calculated. The recoveries of standard uric acid in were between 99.02–101.21%.

Table 2: Validation parameters summery.

Sr. No.	Validation Parameters	Results	Standard Values
1	Linearity Range	0.05 – 30 µg/L	-
2	Straight line equation	Y = 173783[X] + 94483	-
3	Correlation Coefficient	0.9994	≥ 0.9990
4	Precision (% R.S.D.)		
	Repeatability Intraday Interday	0.206 0.398 0.853	≤ 2.0 % R.S.D.
5	Mean % Recovery	100.28	95 – 105%
6	Specificity		Specific
7	LOD (µg/mL)	0.01	-
8	LOQ (µg/mL)	0.033	-
9	Ruggedness	Complies	≤ 2.0 % R.S.D.
10	Robustness		
	Change in Flow rate Change in wavelength Change in pH of buffer	Complies	≤ 2.0 % R.S.D.

Table 3: Accuracy of proposed method for uric acid determination.

Uric acid µg/mL		% Recovery	
Taken	Found		
3	3.018	100.61	Mean = 100.28
5	4.951	99.02	SD = 0.9284
7	7.085	101.21	R.S.D. 0.926

Specificity:

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present. Typically, these might include impurities, degrades etc. A solution of placebo in mobile phase was injected and the chromatogram showed no inferring peaks at retention time of the uric acid. The chromatogram of placebo was compared with those acquired from the uric acid standard solution. The correlation was good (in terms of t_R and area) indicates the specificity of the method. Chromatograms of specificity for the uric acid depicted in (fig. 4 and 5).

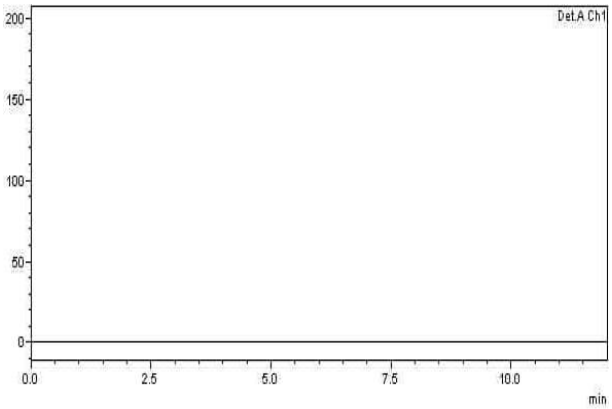

Fig: 4: Specifity chromatogram of blank placebo in buffer: ACN (95:05 v/v).

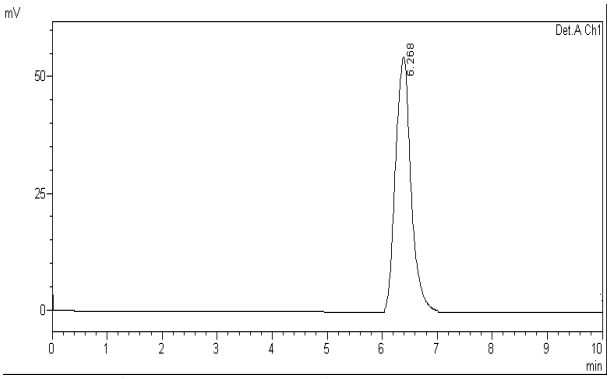

Fig: 5: Specificity chromatogram of standard uric acid (10µg/ L).

Ruggedness:

The ruggedness of the proposed method was determined by analysis of aliquots of sample uric acid solution (7 µg/mL) by two analysts using same operational and environmental conditions. The results obtained are shown in table 4.

Table 4: The ruggedness results of the proposed method.

	Analyst 1	Analyst 2
Mean % Assay* ± SD	99.509 ± 0.5640	98.923 ± 0.5160
% R.S.D.	0.5704	0.5210

* n = 3

Robustness

The robustness of the method were evaluated by changing the flow rate by ±0.1mL/min. (1.1mL/min. and 1.3 mL/min.), changing the mobile phase pH by ± 0.2 % (3.8 and 4.2 %) for buffer and the changing of the detection wavelength by ± 2 nm (283 nm and 287 nm). The results obtained are shown in table 5.

Table 5: The robustness results of the proposed method.

Method Parameters	Mean*	S.D.	%R.S.D.
Flow rate change 1.2 ± 0.1 mL/min.	99.96	0.9117	0.9121
Mobile phase pH change 4.0 ± 0.2	99.58	0.7183	0.7201
Detection wavelength change 285 ± 2 nm	100.21	0.8401	0.8386

* n = 3

Analytical assays:

Four human serum samples were analyzed for uric acid using a validated high-performance liquid chromatography (HPLC) method with UV detection at 285 nm. Serum samples were defrosted at room temperature. Sample preparation was done by liquid phase extraction (0.2 mL of methanol with 0.2 mL of serum. Supernatant layer was separated pre-concentrated and 10µL of sample were injected to HPLC analysis under the optimum separation conditions. Mobile phase compassion buffer solution: ACN (95: 05 v/v) was delivered at a flow rate of 1.2 mL/ min. with UV detection at 285 nm. The column was Phenomenex C-18 (250 × 4.6 mm I.D) and 5 µm particle size. Analysis was performed at room temperature (~25 ^{o}C) and the total run time was 10 min. The results obtained are tabulated

in table 6. Figure 6 and 7 are shown the separation chromatograms of the uric acid in human serum. The recoveries of spiked uric acid in serum samples were between 98.75 – 101.20%.

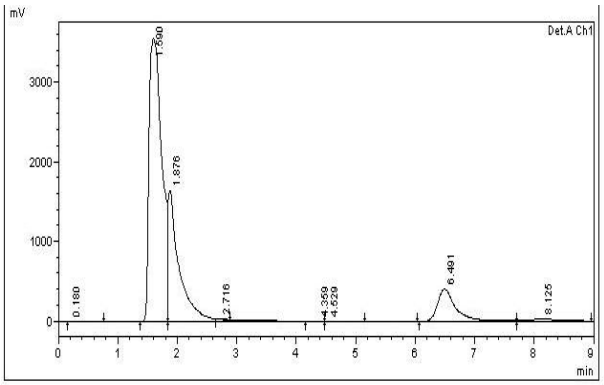

Fig: 6: Separation chromatogram of uric acid in human serum.

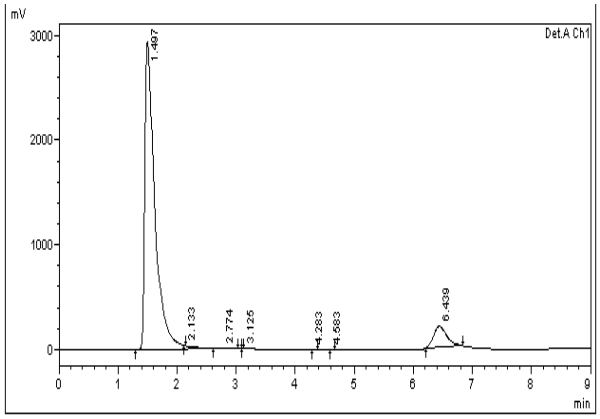

Fig: 7: Separation chromatogram of spiked uric acid in human serum.

Table: 6: Results of uric acid determination in human serum.

Spiked Uric acid µg/mL		% Recovery		Uric acid serum samples µg/mL	
Spiked	**Recovered**			**Sample No.**	**Uric acid found µg/mL**
6	6.072	101.20	Mean = 99.80	1	53
8	7.956	99.45	SD = 1.481	2	32
10	9.875	98.75	R.S.D. 1.478	3	37

CONCLUSION:

The validated RP–HPLC methods employed here proved to be simple, fast, accurate, precise and robust, thus can be used for the routine analysis of uric acid in human serum and in aqueous solution.

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AUTHORS' CONTRIBUTIONS:

All the authors have contributed equally.

CONFLICT OF INTERESTS:

Declared none.

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