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The optimal method to measure polyamines in serum by using HPLC fluorescence detector

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ABSTRACT

Measurement of polyamines (putrescine, spermidine and spermine) is important in the monitoring of many metabolic events, as well as in cancer prognosis. However, the lack of method standardization and validation for the measurement of polyamines is a major obstacle. In this study, sample preparation, gradient program, chromatography column, sample concentration studies were attempted and an analysis method was developed for the detection of polyamines in serum by using HPLC fluorescence detector in order to reduce the difficulties experienced with the existing methodologies. This method was validated analytically in accordance with EMA and FDA's guidelines. In this study, Waters Nova-Pak C18 A chromatography column with 3.9 mm, 150 mm and 4 μm specifications was used for the first time. Acetonitrile was used instead of methanol. Thanks to the newly developed gradient, peak purity and sufficient peak separation were ensured and accurate, sensitive, reliable and reproducible method was developed and validated.

Keywords: Polyamines, HPLC, derivatization, O-Phthalaldehyde, N-Acetyl-L cysteine

INTRODUCTION

Polyamines are involved in functions related to cell growth and differentiation, such as DNA synthesis and stability, regulation of transcription, ion channel reg-

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ulation and protein phosphorylation. Their role in cancer metabolism and gut maturation, as well as their antioxidant properties and aging are being investigated. Natural polyamines putrescine (Put), spermidine (Spd), and spermine (Spm) are found in all living cells. The biosynthesis of polyamines in mammalian cells begins with putrescine synthesized from L-ornithine. Spermidine is synthesized from putrescine and spermine, from spermidine[1]. Polyamines (putrescin, spermidine, and spermine) are simple aliphatic primary amines that are fully protonated under physiological conditions, essential components of eukaryotic and prokaryotic cells. They are involved in performing many cell functions including cell growth and differentiation and receptor function. They also affect DNA replication, gene expression, protein synthesis, stabilization of lipids, brain development, nerve growth and regeneration [2]. Excessive production or excessive intake of polyamines is toxic to cells and facilitates cell death by oxidative mechanism [3]. These compounds were also suggested as possible tumor markers [4-7].

Because of its recognized importance in clinical diagnosis, it has become a necessity to produce inexpensive, easy, and reproducible methods of polyamine measurement. Various methods for the analysis of polyamines were studied for many years. Thin Layer Chromatography (TLC), enzymatic assay method, benzoyl chloride and dansyl chloride derivatization methods of polyamines using HPLC UV detector were used for the detection of amines [8-14].

The losses experienced in the sample preparation stage in derivatization methods and some limitations in applications cause serious difficulties in terms of obtaining reproducibility and accurate results [15].

LC-MS systems are systems which offer the most selective, sensitive and reproducible results compared to UV and Fluorescent systems. With the development of MS systems in parallel with technological developments, studies were carried out with HPLC/Quadrupole-Time of Flight (Q-TOF)-MS systems. Liu et al. proposed a method to detect the amounts of polyamines in human plasma through benzoyl chloride derivatization process using the HPLC/Q-TOF-MS system [16].

EMA and FDA are currently publishing guidelines for the validation of biological fluids, and these guidelines are used for analytical method validation of biological fluids[17]. Method validity of biological fluids contain the parameters of accuracy, precision, linearity, selectivity, lower limit of quantification (LLOQ), recovery, intra-day and inter-day repeatability and inter-day repeatability [18, 19].

In this study, we aimed to develop a method for the measurement of polyamine in serum, analytically validated with EMA and FDA guidelines by using HPLC fluorescence detector.

METHODOLOGY

Demographics of the Patient and Control Groups

In this study, healthy individuals who came to Medipol University Mega Hospital Laboratory for health checks were informed regarding the study and their blood was taken after receiving their written consent. 40 healthy control serums were obtained from selected individuals according to the exclusion criteria.

Exclusion criteria were determined as being younger than 18 years of age, older than 40 years of age, smoking and use of alcohol, having kidney function disorders, hypertension, heart disease, osteoarthrosis, diabetes, obesity, cancer, polycystic ovarian disease, inflammatory and infectious diseases.

Collection and Storage of Blood Samples

After 12 hours of fasting,10 ml of venous blood was taken from 40 healthy control groups, which was then incubated for 30 minutes at room temperature in vacuum gel tubes and left to coagulate. After coagulation, the tubes were incubated at 1200 g for 15 minutes at room temperature, following which they were centrifuged, and serum separated. For other tests that were not studied on the same day, the sera were transferred to Eppendorf tubes and stored at -80˚ C until the day of study.

Parameters and Methods Examined in Blood Samples

Polyamines levels (putrescine, spermidine and spermine) measured in the REMER laboratory using the HPLC method. Initially, a serum pool was created from the 40 serum samples collected. Selectivity, calibration/standard curve, accuracy, precision, intra-day and inter-day repeatability, recovery, lower limit of test (LLOQ) and stability parameters were performed as specified in the EMA and FDA validation guidelines[18, 19].

Devices and Equipment Used

Chemicals

The following chemicals were used as Merck; Sodium Acetate Trihydrate, Hydrochloric Acid Fuming 37%, Acetonitrile, Potassium Carbonate and Sodium Tetraborate Decahydrate. The following chemicals were used as Sigma Aldrich; Methanol, Tetrahydrofuran, Perchloric Acid, Benzoic Acid, Potassium Tetraborate Tetrahydrate, O-Phthalaldehyde, N-acetyl-L-cysteine, Brij-35 Solution (Brij-L23), Putrescine-2HCI, Spermidine-3HCl and Spermine-4HCI.

Instruments, System Setup and Chromatographic Requirements

The HPLC system was a Waters Alliance 2695 HPLC system equipped with an analytical column (Waters Nova-Pak C18 3.9mm*150mm*4µm). A Waters 2475 fluorescence detector was used to detect the fluorescence each polyamine derivative, with excitation λ set at 340 nm and emission λ at 450 nm. The HPLC conditions for the of separation were as follows; mobile phase A was (0.1 M sodium acetate; pH 7.2): added 27.3 g sodium acetate (trihydrate) and 96 µl of 6 N HCl to 1.6 l LC grade water and on this solution add 180 ml methanol and 10 ml tetrahydrofuran. The final volume was made up of 2 l with HPLC grade water. The solution was mixed thoroughly. Mobile phase B was LC grade acetonitrile. The HPLC was run at a gradient program. The flow rate was 1.0 mL/ min, with the following gradient: 95%-A, 5%-B to 73%-A, 27%-B in 20 min; 73%-A, 37%-B to 30%-A, 70%-B in 1 min; 30%-A, 70%-B from 21 to 25 min; returning to 95%-A, 5%-B from 25 to 26 min and the column was conditioned between 26 and 30 minutes. The total running time for each sample (including column regeneration on the automated system) was 30 minutes. Column temperature was maintained at 40 °C and autosampler temperature was maintained at 4°C. The in-line pre-column derivatization of polyamines was accomplished by setting the "auto addition" option in the sample set method of the software Waters Empower 3. A volume of 10 µl derivatization + 10 µl blank, standards solutions, sample solutions was injected. After mixing in the derivatization loop, an injection (a total volume of 20 µl) was automatically performed with no delay. Separated polyamines were monitored and peak integration and calculations of concentrations against the standard curve were performed using the Waters Empower 3 software, recording and analyzing the HPLC chromatography data.

Preparation of Standard and Sample Solutions

Preparation of 100 ng/ml Polyamine Standard Solution Mixture: Standard amounts of 18.3 mg putrescine-2HCI, 17.5 mg spermidine-3HCl and 17.2 mg spermine-4HCl were precisely weighed and filled into a 10.0 ml plastic balloon flask. Then 5 ml of deionized water was added, and the mixture was was vortexed thoroughly for 2 minutes. Then the mixture was kept in a cold ultrasonic bath for 1 minute, following which the solution was completed to its volume using deionized water. Then we waited for the mixture temperature to reach ambient laboratory conditions. 10.0 µl of this solution was taken into a 1.5 µl eppendorf tube and 990 µl of deionized water was added to it and mixed in vortex for 30 seconds. 10.0 µl of this solution was transferred to a 1.5 ml Eppendorf tube. 940 µl of deionized water and 50 µl of 1.2% (w/v) benzoic acid solution were added and mixed in vortex for 30 seconds. The solution was stored at 0-4 ˚C until used [16, 17].

Sample Solution Preparation

The serum sample pool stored at -80 ˚C were dissolved in laboratory conditions and 100 µl of each solution was taken into a 1.5 ml eppendorf tube. 250 µl of deionized water was added and mixed with vortex for 10 seconds. 100 µl of cold 1.5 M $HCIO_4$ was added to this solution and mixed in vortex for 30 seconds. A cold 50 µl of 2 M K_2CO_3 was added and mixed in vortex for 30 seconds. Tube cap was opened, and gas was released for 20 seconds. Then the solution was centrifuged for 3 minutes at 14000 g and 4 ˚C. 200 µl of the supernatant liquid was transferred to a 1.5 ml Eppendorf tube. 750 µl of deionized water and 50 µl of 1.2% (w/v) benzoic acid solution were added and mixed in vortex for 30 seconds. This solution was transferred to the HPLC vial and then injected [16].

Preparation of Polyamine Spiked Sample Solutions

Preparation of Stock Standard Solution: The solution was prepared as in 100 ng/ml Polyamine standard solution mixture. 25.0 µl of this solution was taken into a 1.5 µl eppendorf tube and 975 µl of deionized water was added to it and mixed in vortex for 30 seconds.

Spike Sample Solution Preparation: The sera of 6 healthy individuals stored at 80 ˚C were dissolved in laboratory conditions and 100 µl of each was taken into a 1.5 ml eppendorf tube. 240 µl of deionized water was added and mixed in vortex for 10 seconds. On top of this solution, 10.0 µl of the stock standard solution and 100 µl of cold 1.5 M $HCIO_4$ were added and mixed in vortex for 30 seconds. Cold 50 µl of 2 M K_2CO_3 was added and mixed in vortex for 30 seconds. Tube caps were opened to enable gas escape. Then the solution was centrifuged for 3 minutes at 14000 g and 4 ˚C. 200 µl of the supernatant liquid was transferred to a 1.5 ml Eppendorf tube. 750 µl of deionized water and 50 µl of 1.2% (w/v) benzoic acid solution were added and mixed in vortex for 30 seconds [16, 17].

Validity Tests of the HPLC Method

The validity test of the HPLC method to be used in the determination of polyamine in serum samples in this study was performed based on the parameters of selectivity, calibration/standard curve, accuracy, precision, intra-day repeatability, inter-day reproducibility, recovery, LLOQ and stability specified in EMA and FDA validation guidelines [18,19].

Selectivity

In the HPLC method, to show that the chemicals in the mobile phase, serum matrix, other impurities and standard peaks do not interfere these solutions were injected separately into the HPLC device calibrated according to polyam-

ine standards. Chromatograms of peak areas versus retention times of the solutions were obtained. With the help of PDA detector, the spectra of the injections were taken between 190 nm and 800 nm. Peak purity was determined from the obtained spectra.

Calibration/Standard Curve (Linearity Range) Study

The linearity of the method was determined through repeated analyzes of 8 standards at each concentration, including the LLOQ, in the concentration range of 0.1-200.0 ng/ml. While selecting the working range in both studies, concentration ranges where acceptable accuracy, precision and linearity were obtained were preferred. For the calibration standards to be used during the study, the concentrations are given in Table 1; 8 standards, including putrescine, spermidine, and LLOQ of spermine at 10.0 ng/ml, 50.0 ng/ml, 80.0 ng/ml, 100.0 ng/ml, 150.0 ng/ml, 200.0 ng/ml and 250.0 ng/ml solution was prepared. All solutions were labeled and stored at -20 ˚C in the dark. Solutions were diluted by dissolving in laboratory conditions before each study. In the linearity study, 3 injections for each were studied and the average was calculated, and the linearity graph was drawn. Calibration curves were derived by plotting the peak area ratios obtained against the concentration of the solution in the concentration ranges specified in the study (n=3). Correct equations of the standard curve and correlation coefficients were obtained by regression analysis of the calibration curves.

Table 1: Concentrations of calibration standards

Solution Level	Concentration of Putrescine (ng/ml)	Concentration of Spermidine (ng/ml)	Concentration of Spermine (ng/ml)
LLOQ	0.2	0.5	1.0
10.0%	10.0	10.0	10.0
50.0%	50.0	50.0	50.0
80.0%	80.0	80.0	80.0
100.0%	100.0	100.0	100.0
150.0%	150.0	150.0	150.0
200.0%	200.0	200.0	200.0
250.0%	250.0	250.0	250.0

Accuracy/Precision Study

Quality control solutions were prepared at three different concentrations (50.0, 100.0 and 150.0 ng/ml) corresponding to the concentration range determined in the linearity study. These solutions were analyzed 6 times in 1 day under the same laboratory conditions using the same method for intraday reproducibility analyses, and 6 times in 3 consecutive days, using the same method for intraday reproducibility analyses, under the same laboratory conditions. Accuracy and precision values were calculated from the obtained results. The mean, standard deviation (SD), relative standard deviation (%RSS) and relative errors (%RE) of the analysis results were determined. Accuracy was given with % relative error (%RE) and precision with % relative standard deviation (%RSS).

Recovery Study

Recovery experiments were performed using standard addition method, comparing analytical responses at three different concentrations (low, medium, high) with non-extracted standards (representing 100% recovery). For recovery, 3 parallel samples of each concentration were prepared at three different concentrations and each sample was analyzed 3 times. Theoretical concentrations (Calculated Concentration) were calculated using the concentration curve equation related to the areas obtained from the injection of the recovery samples, versus the standard areas obtained from the linearity range chromatogram. Theoretical concentrations (added concentration) of the standards added on the samples by the standard addition method were calculated.

Calculation was made using the formula % Recovery = (Calculated Concentration/Added Concentration) x100. Recovery solutions were prepared as serum polyamine concentrations of 50, 100 and 150 ng/ml.

Quantity Determination Lower Limit Study (LLOQ)

As a rule of thumb, the signal-to-noise (S/N) ratio was used in chromatographic measurements. The analyte response on the LLOQ was determined to be at least 5 times the response compared to the blanked response. The analyte peak (response) has been shown to be identifiable, discrete, and reproducible with an accuracy of 20% and an accuracy of 80-120%.

LLOQ values for putrescine, spermidine and spermine were obtained from linearity study. The following conditions were fulfilled in the development of the linearity curve.

- 20% deviation of LLOQ from nominal concentration

- 15% deviation of standards other than LLOQ from nominal concentration

At least four of the six nonzero standards met the above criteria, including the LLOQ and the calibration standard at the highest concentration.

Stability

The stability study was performed on serum samples spiked with three different levels of polyamine. The following methods were used for stability determination.

Short Term Temperature Stability

Three aliquots of each analyte at low, medium, and high concentrations were dissolved at room temperature. They were stored under refrigerator (+4 ˚C) storage conditions and analyzed at 0, 2, 4, 8, 12 and 24 hours.

RESULTS and DISCUSSION

EMA and FDA are currently publishing guidelines for the validation of biological fluids, and these guidelines are used for analytical method validation of biological fluids[17]. In the analytical method validation of the method developed considering the parameters specified in these guidelines, selectivity, linearity, accuracy, precision, recovery, intra-day and inter-day reproducibility, LLOQ and solution stability studies were performed. HPLC methods are widely used due to their high sensitivity and repeatability and ease of automation. Our goal is to separate and quantify polyamines using reverse phase chromatography [18]. HPLC technique is the development of a simple, fast, precise, accurate and precise method. The purpose of all of these studies is to develop and validate a method which is usable in clinical practices as a cheap, fast, reproducible and fast. For this purpose, pre-column in-line derivatization method was preferred by using fluorescent detector and o-phthalaldehyde-N-acetyl-L-cysteine reagents in HPLC system.

HPLC Method Validation Tests

Selectivity Study

No other peaks were observed at the retention time of the polyamine peaks and the peak purity was determined accordingly. No interference was found in the retention times of the polyamine peaks in the chromatograms taken under the chromatographic conditions determined as a result of method development studies.

In the selectivity study, the retention times obtained as a result of the injection of the standard solution with a 30-minute gradient program were approximately 19.2 minutes for putrescine and 18.0 minutes for spermidine and 17.1

min for spermine. The purity angle and purity threshold values obtained from the Empower 3 program were 0.570 and 1.103 for putrescine, 0.361 and 0.406 for spermidine, and 0.361 and 0.406 for spermine. In the method developed according to these data, the peak purity is appropriate, and the peaks were obtained pure. As a result of the 30-minute gradient program they applied in the method, Dai et al. found the retention times to be approximately 12.2 minutes for putrescine, 11.1 for spermidine, and 14.1 for spermine [20].

Calibration/Standard Curve (Linearity Range) Study

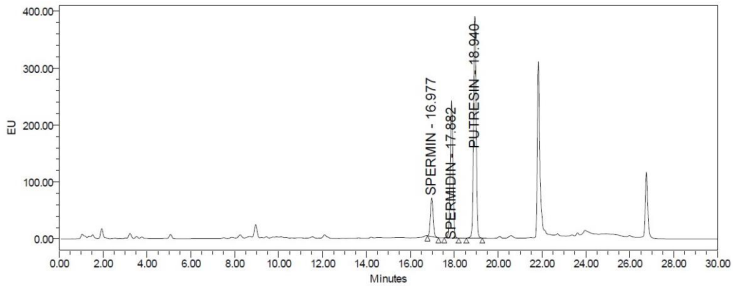
Chromatograms were taken for each of 8 standards, including LLOQ, at concentrations of 0.2-250 ng/ml for putrescine, 0.5-250 ng/ml for spermidine and 1.0-250 ng/ml for spermine, and peak areas were determined. Obtained by HPLC method; Putrescine, spermidine and spermine solution linearity results and statistical analysis values of calibration curve are given in Table 2.

Table 2. Linearity study results of Putrescine, Spermidine and Spermine

Solution Levels	Putrescine Concentration (ng/ml)	Spermidine Concentration (ng/ml)	Spermine Concentration (ng/ml)
LLOQ	0.2	0.5	1,0
10.0%	9,9	10,2	10,0
50.0%	49,6	51,1	50.0
80.0%	79,4	81,7	80.0
100.0%	99,2	102,2	100.0
150.0%	148,8	153,3	150,0
200.0%	198,4	204,4	200.1
250.0%	248.0	255,4	250.1
Linearity Range (ng/ml):	0.2-250.0	0.5 - 250.0	1.0 - 250.0
Slope:	211992.7343	136673.7451	44695.4958
Intercept:	- 163096.5089	- 619833.0382	- 389224.3886
Correlation Coefficient (r^2):	0.9996	0.9981	0.9937

Calibration curves were created by plotting the peak areas obtained against the solution concentrations of putrescine, spermidine and spermine. The correlation coefficient (r^2) found by the least squares method should be ≥0.9950. The r^2 value for putrescine, spermidine, and spermine were 0.9996, 0.9981, 0.9937,

respectively. According to these results, the method; linear for putrescine, spermidine, and spermine. A respective example linearity chromatogram is given below (Figure 1).



	Peak Name	RT	Area	Height
1	SPERMIN	16.977	5902242	680318
2	SPERMIDIN	17.882	20079893	2406922
3	PUTRESIN	18.940	31440893	3912837

Figure 1. Chromatogram of 150% linearity standard

Linearity limits including LLOQ for putrescine, spermidine, and spermine were 0.1-250 ng/ml, 0.5-250 ng/ml, 1-250 ng/ml, respectively. Correlation values were found as 0.9998, 0.9990 and 0.9968, respectively. Liu et al. found linearity limits for each polyamine as 0.4-200.0 ng/ml and correlation values as 0.9941, 0.9939 and 0.9937, respectively [16]. The values found as a result of our study meet the criteria specified in the FDA and EMA validation guidelines [18,19]. LLOQ values for putrescine, spermidine, and spermine were found as 0.2 ng/ml, 0.5 ng/ml and 1.0 ng/ml respectively. Liu et al. found the lower limit of quantitation values in the range of 0.02-0.1ng/ml [16].

Accuracy/Precision Study

Intraday accuracy values for putrescine, spermidine and spermine were 6.3-13.6%, 3.4-8.6%, 1.5-6.0%, respectively. Liu et al. found the intraday accuracy values to be 7.8-9.3%, 5.0-14.0%, 3.9-7.9%, respectively [16]. Intraday precision values for putrescine, spermidine, and spermine were 0.9-1.7%, 0.2-0.3%, and 0.1-0.7%, respectively.

Inter-day accuracy values for putrescine, spermidine, and spermine, were found to be 5.7-12.4%, 6.1-9.1%, 4.5-12.6% respectively. Liu et al. found the intraday accuracy values to be 4.3-9.8%, 7.3-11.1%, and 2.9-3.5%, respectively [16]. Inter-day precision values for putrescine, spermidine and spermine were 6.2-9.7%, 1.6-6.1%, 5.4-13.8%, respectively.

According to FDA criteria, intraday and interday accuracy and precision values should be below 15%[18]. In this study, all values were found below 15% and all criteria were met. Results obtained by HPLC method are given in Table 3 for intra-day and inter-day accuracy and precision values.

Table 3. Intra-day and Inter-day accuracy and precision values

		Intra-day accuracy and precision values			Inter-day accuracy and precision values		
Active Ingredient	**Added Conc. (ng/ml)**	**Calculated Conc. ±SD (ng/ml)**	**RE % (Accuracy)**	**%RSS (Precision)**	**Calculated Conc. ±SD (ng/ml)**	**RE % (Accuracy)**	**% RSS (Precision)**
Putrescine	50	42.6 ±0.4	13.6	0.9	52.7 ±5.1	12.4	9.7
	100	86.1 ±1.5	12.8	1.7	96.0 ±5.9	5.7	6.2
	150	138.7 ±1.3	6.3	0.9	144.6 ±12,9	6.8	8.9
Spermidine	50	49.4 ±0.2	3.4	0.3	49.8 ±3.0	6.1	6.1
	100	93.4 ±0.3	8.6	0.3	87.2 ±1.4	9.1	1.6
	150	147.5 ±0.5	3.8	0.3	128.4 ±3.4	8.5	2.6
Spermine	50	49.9 ±0.06	1.5	0.1	52.6 ±4.5	8.0	8.6
	100	95.3 ±0.7	6.0	0.7	92.1 ±4.8	4.5	13.8
	150	148.6 ±0.3	2.1	0.2	124.3 ±6.7	12.6	5.4

Conc.: Concentration, SD: Standard Deviation, %RSS: Percent Relative Standard Deviation, %RE: Percent Relative Error

Recovery Study

Recovery values were 89.55% for putrescine, 89.62% for spermidine and 88.36% for spermine. Liu et al. found the recovery values to be 80.6% for putrescine, 79.5% for spermidine and 84.0% for spermine [16]. Although 100% recovery of the analyte is required according to FDA guidelines, the degree of recovery of an analyte and internal standard must be consistent, precise, and reproducible [18]. In the study, a high recovery of nearly 100% was achieved and consistent, precise and reproducible results were obtained. These results show that the recovery degree of the method is appropriate. Recovery study results are given in Table 4 for putrescine, Table 5 for spermidine, and Table 6 for spermine.

Table 4. Putrescine recovery study results

Concentration Level	Added Concentration (ng/ml)	Calculated Concentration (ng/ml)	Recovery %
50%	49.33	47.00	95.28
	49.33	47.44	96.17
	49.33	43.45	88.08
100 %	98.67	87.63	88.82
	98.67	82.82	83.93
	98.67	85.86	87.02
150%	148.00	133.24	90.03
	148.00	128.82	87.04
	148.00	132.64	89.62
Average % Recovery Value (ng/ml):		89.55	
Recovery 95% Confidence Interval Limits (ng/ml):		86.53-92.57	
Standard Deviation (ng/ml):		3.93	
Relative Standard Deviation (%):		4.39	

Table 5. Spermidine recovery study results

Concentration Level	Added Concentration (ng/ml)	Calculated Concentration (ng/ml)	Recovery %
50%	51.09	52.31	102.39
	51.09	52.22	102.21
	51.09	48.90	95.71
100 %	102.18	88.13	86.25
	102.18	83.82	82.03
	102.18	86.81	84.96
150%	153.27	130.83	85.36
	153.27	126.93	82.82
	153.27	129.98	84.81
Average % Recovery Value (ng/ml):		89.62	
Recovery 95% Confidence Interval Limits (ng/ml):		83.32-95.92	
Standard Deviation (ng/ml):		8.19	
Relative Standard Deviation (%):		9.14	

Table 6. Spermine recovery study results

Concentration Level	Added Concentration (ng/ml)	Calculated Concentration (ng/ml)	Recovery %
50%	51.46	53.65	104.26
	51.46	49.62	96.43
	51.46	46.71	90.77
100 %	102.92	84.82	82.42
	102.92	81.06	78.76
	102.92	84.79	82.38
150%	154.38	129.61	83.95
	154.38	134.40	87.06
	154.38	137.73	89.22
Average % Recovery Value (ng/ml):		88.36	
Recovery 95% Confidence Interval Limits (ng/ml):		82.23-94.49	
Standard Deviation (ng/ml):		7.98	
Relative Standard Deviation (%):		9.03	

Lower Limit of Quantification (LLOQ) Study

The LLOQ values of the method were determined as 0.2 ng/ml for putrescine, 0.5 ng/ml for spermidine and 1.0 ng/ml for spermine.

Stability Study

For short-term temperature stability study, serum samples with three different levels of polyamine spiked were prepared as described in section stability. They were stored at 4˚C and analyzed at 0, 2, 4, 8, 12 and 24 hours. Areas were determined for each level. The % changes with baseline areas were determined for each study. At the end of the 24th hour, the % change was found to be 9.1% for putrescine, 9.9% for spermidine and 9.8% for spermine. According to these results, solutions of putrescine, spermidine and spermine are stable for 24 hours at 4 ˚C.

As a result, a sensitive, reproducible and reliable method was developed and validated for routine analysis of serum samples in clinical laboratories. This work can be extended to make it applicable to all biological fluids and can be applied for routine analysis.

ETHICAL STATEMENT

Our study was approved by Medipol University local ethics committee (Ethical approval no: 277, date: 28.07.2017)

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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AUTHOR CONTRIBUTIONS

These authors contributed equally.

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