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DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR THE DETERMINATION OF NITRITE AND ITS APPLICATION IN THE BIOLOGICAL FLUIDS OF PATIENTS

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Abstract

Purpose of study: The study's goal was to measure nitrite using the high-performance liquid chromatography (HPLC) technique in numerous biological fluids i.e. serum, ascetic fluid, and cerebrospinal fluid (CSF).

Methodology: Using distilled water (pH 6.5 with H_3PO_4) as the mobile phase and a flow rate of 0.9 ml per min., an HPLC method was developed for the quantitative determination of nitrite in serum,

CSF, and ascetic fluid. With a nitrate retention time of 3.5 minutes, the eluate was detected at 220 nm. The LOD and LOQ values of nitrite were 0.006 µg/ml and 0.012 µg/mL, correspondingly. A 250 × 4.6 mm, i.d. C18 BDS Hypersil column containing particles with a size of 5 µm was used to elute nitrite. Ten minutes was the run time. The method was validated in compliance with FDA guidelines, and the results showed that the % CV was less than 3% and the method was linear in the range of 0.012 to 100 µg/mL.
Conclusion: The developed technique was successfully used to measure the amount of nitrite in biological fluids, including serum, CSF, and 20 patients' ascetic fluids. The work discusses the results.

Keywords: Biological Fluids, Cerebrospinal Fluid, Ascitic Fluid, HPLC, Nitrites.

INTRODUCTION

A useful mediator of physiological and pathological processes is nitric oxide (NO). It is produced in a variety of tissues and cells and functions as a neurotransmitter, vasodilator, and killer molecule in the immune system [1]. *In vivo*, NO has a very brief half-life of roughly 0.1 seconds. Nitrite and nitrate, two stable products, are the end products of NO metabolism [2]. The gradual oxidation to nitrite and nitrate is the most significant pathway for NO metabolism. NO is oxidized to nitrite in plasma and other physiological fluids or buffers, where it stays stable for numerous hours [3]. Reactive free radicals, or NO, are formed when nitrites, nitrates, nitroso compounds, and other compounds containing nitrogen oxide are present. NO has the ability to stimulate guanylyl cyclase, raise cyclic GMP levels in the cell, which in turn activates PKG, the cyclic GMP-dependent protein kinase, and alter the activities of PDEs 2, 3, and 5 (cyclic nucleotide phosphodiesterases) in a variety of cell types [4]. Reduced myosin light chain phosphorylation, decreased cytosolic Ca^{2+} concentration, and relaxation are the overall effects in smooth muscle. The NO-mediated elevation of intracellular cyclic GMP has a significant effect on the cyclic GMP-dependent protein kinase, which is responsible for the phosphorylation of multiple proteins in smooth muscles. Another important target of this kinase is myosin light chain phosphatase, which is activated upon binding cyclic GMP-dependent protein kinase and causes the light chain myosin chain to be dephosphorylated, thereby promoting vasorelaxation. [5]. The arginine–nitric oxide pathway can also produce nitrite, but it would be imperceptible due to its quick oxidation to nitrate [6].

Methemoglobinemia in infants with diarrhea is one possible example of nitrite formation through this route [7]. Nitrate is an important signaling molecule in and of itself and a crucial homeostatic molecule in NO biology. Recently, nitrite has garnered a lot of attention because of its capacity to directly nitrosate thiols to form RSNOs and, under certain physiological conditions, its ability to be reduced back to NO. [8].

Recent research has made it possible to use the molecule nitrite, which is important to physiology, for both therapeutic and diagnostic purposes. This is particularly true when it comes to cardiovascular illnesses, as nitrite can be used as a marker as well as an active ingredient. Moreover, it has been shown recently that the food ingredient nitrate lowers the diastolic blood pressure of healthy volunteers [9]. Thus, all nitrite and nitrate in biological samples should be handled carefully.

METERIALS AND METHODS

Materials

We bought sodium nitrite from Fluka, Switzerland. We bought HPLC-grade acetonitrile, methanol, and sodium nitrate from Merck in Darmstadt, Germany. HPLC-grade ingredients included phosphoric acid, hydrochloric acid, and de-ionized double-distilled water.

Method Development

Nitrite in the serum, CSF, and ascitic fluid can be measured using an HPLC technique that has been developed and standardized. Multiple systems of the mobile phase with multiple mixtures were tested during the development process. At a flow rate of 1.0 mL/min, it was discovered that distilled water with a pH of 6.5 was the most appropriate. A 250 × 4.6 mm, i.d. C18 BDS Hypersil column containing particles with a size of 5 µm was used to elute nitrite. Every elution had a 10-minute run time, and it was detected at 220 nm. 0.006 µg/mL was the LOD and 0.012 µg/mL was the LOQ. The linearity of the method was observed between 0.012 µg/mL and 100 µg/mL.

Preparation of mobile phase

Water was distilled using an IRMECO GmBH water distillation apparatus to create the mobile phase, which was subsequently filtered through a 0.45 µm membrane filter. To bring the pH down to 6.5, orthophosphoric acid was added. Following filtration, sonication, and degassing, mobile phase was used.

Stock solutions preparation

Nitrite stock solution (1.0 mg/ml) was made with distilled water. Dilutions were prepared all the way up to 0.006 µg/mL. Every day, fresh solutions were made, filtered, and sonicated to remove gas.

Peak recognition and retention duration

The size of the nitrite peak increased or decreased in response to changes in the concentration of the reference solution, indicating and validating its identification. The nitrite retention time during this method's application was 3.5 minutes. Figure 1 is a typical chromatogram showing the retention time of nitrite with provided mobile phase.

Preparation of Samples of biological fluids

For the purpose of determining nitrite, serum was diluted five times, CSF three times, and ascetic fluid four times.

HPLC method validation for determination of nitrite

The nitrite determination procedure mentioned above underwent a phased validation process to ensure that important method characteristics were tested in compliance with good analytical practice guidelines.

Linearity and range

Linearity is the analytical method's capacity to yield test results that are exactly proportionate to the sample concentration and fall inside a given range. The relationship between the known concentrations of pure drug samples and the instrumental response was ascertained by plotting the calibration curve. A series of solutions with varying concentrations ranging from 0.006 to 100 µg/mL were created by diluting the standard stock solution with mobile phase (Table 1-2). In the mobile phase, the nitrite calibration curve (98.00% purity) was created. To determine the calibration curve's parameters, every sample was run three times, and the data was contrived (Figure 2). For nitrite, the corresponding values of slope, intercept, and r-square were 6.54, 2.35, and 0.999. It was discovered that the values of these parameters complied with FDA method validation guidelines.

Accuracy

The accuracy of an analytical method indicates how closely test results agree with the mean value. Accuracy was determined by selecting three nitrite concentrations in triplicate: low, medium, and high. The mean accuracy values for pure nitrites were 101.30%, 102.63%, and 99.17% for low, medium, and high, respectively (Table 3 and 4).

Precision

To guarantee the accuracy of this analytical method, elution was carried out in triplicates at three different concentrations (low, medium, and high). Three separate days were used to repeat this assay. Tables 3 and 4 showed that the precision is within FDA guidelines.

Sensitivity

Table 5 shows that the nitrite's LOD and LOQ values were 0.006 and 0.012 µg/mL, respectively.

Robustness

The method demonstrated robustness under a range of conditions, including flow rate (±0.05 ml/min), mobile phase compositions (±0.5 pH variation), and wavelength (±6-8 nm), with no noticeable changes observed in the elution profiles.

Application of method

This developed technique was used to measure the nitrite levels in ascitic fluid, CSF, and serum. These outcomes are displayed in Figure 3 and Tables 7, 9, and 11.

RESULTS AND DISCUSSION

The study aimed to develop and validate analytical techniques for the measurement of nitrite in different biological fluids. Standard curves were designed and assayed in triplicate in order to evaluate the linearity, accuracy, precision, sensitivity (LOD and LOQ), and stability of the method. Mobile phases with various compositions and combinations of organic and aqueous phases were tested during the development process. Water at pH 6.4 was the last mobile phase to be chosen for the nitrite measurement. Following extensive trial and error, the flow rate was set at 0.9 mL/min. There was 3.5 minutes of retention. The characteristics listed in the results section—linearity, accuracy, precision, stability, sensitivity, and robustness—were used to standardize and validate the analytical methods. The developed technique proved to be straightforward, sensitive, and stable when it was successfully used to measure nitrite in biological fluids. Numerous techniques for measuring nitrate and nitrite separately or simultaneously in different biological fluids have been developed and documented. An HPLC method was reported by Murad and Korenaga [10] to find out how much nitrate and nitrite are in saliva. $NaHCO_3$ at 0.3 mM and Na_2CO_3 at 2.7 mM were used. The flow rate was 1.5 ml/min, and the detection method was a conductivity detector. The LOD and LOQ of the procedure were 15.0 µg/L and 33.5 µg/L, respectively.

The analytical column that was used was the Ion Pac AS12A (200x4).A reliable technique for determining the amount of nitrite and nitrate in cell culture medium was presented by Tahboub [11]. The underlying idea of this procedure was to produce 2,3-naphthotriazole (NAT) by pre-column derivatization of nitrite with 2,3-diaminonaphthalene (DAN) in an acidic medium. A 10 µm C18 column (20X4.6 mm, id) protected a 5 µm C18 column (250X4.6 mm, id) used for NAT separation. A 15 mM phosphate buffer (pH 7.5) containing 35% acetonitrile was utilised as the mobile phase, with a flow rate of 1.0 mL/min. The fluorescence of NAT was observed at 375 nm for excitation and 415 nm for emission, while its absorbance was measured at 369 nm. This method's linearity fell between 0.03-2.0 µM for UV-VIS detection and 0.5-50 µM for fluorescence detection. The mobile phase contained 1.7 mM NaHCO3 and 1.8 mM Na2CO3, and the column utilised was an IonPac As9-SC ion exchange column (250x4). 214 nm was the detection wavelength, and the flow rate was 1.5 mL/min. The process was linear in the 0.2–100 µm range. The LOD for nitrate was 0.5 µm, and the LOD for nitrite was 0.1 µM. Ascitic fluid, CSF, and serum were directly tested for nitrite in the current study. The method that was devised was sensitive and verified. The method's linear range was found to extend from 0.012 µg/mL to 1000 µg/ml. The corresponding slope, intercept, and r-square values for nitrites were 6.542, 2.352, and 0.999. The mean accuracy values for nitrite were 101.3%, 102.6%, and 99.17% for low, medium, and high, respectively. For low, medium, and high nitrite concentrations, the percentage CV within batch was 2.99, 1.76, and 1.01, and the percentage

CV between batches was 2.76, 2.9, and 1.16. The LOD and LOQ values of the nitrites were 0.006 µg/mL and 0.012 µg/ml, respectively. For 3.5 minutes, nitrite was held in reserve.

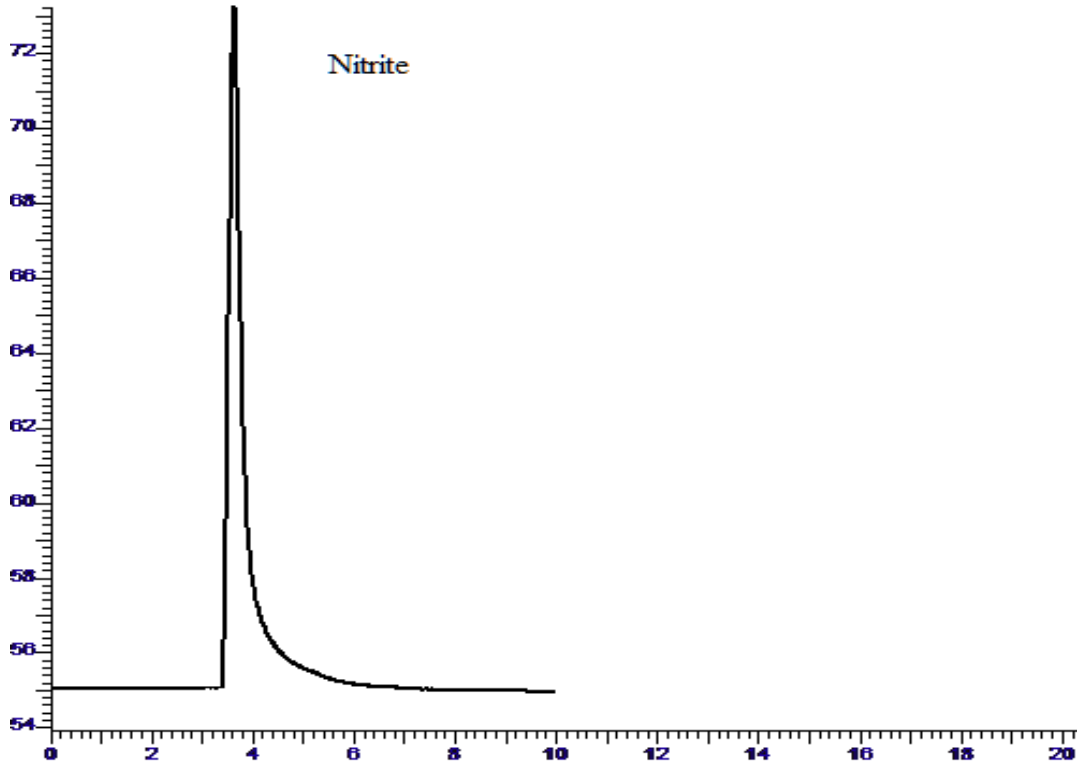

Figure 1. A chromatogram of nitrite elution profile.

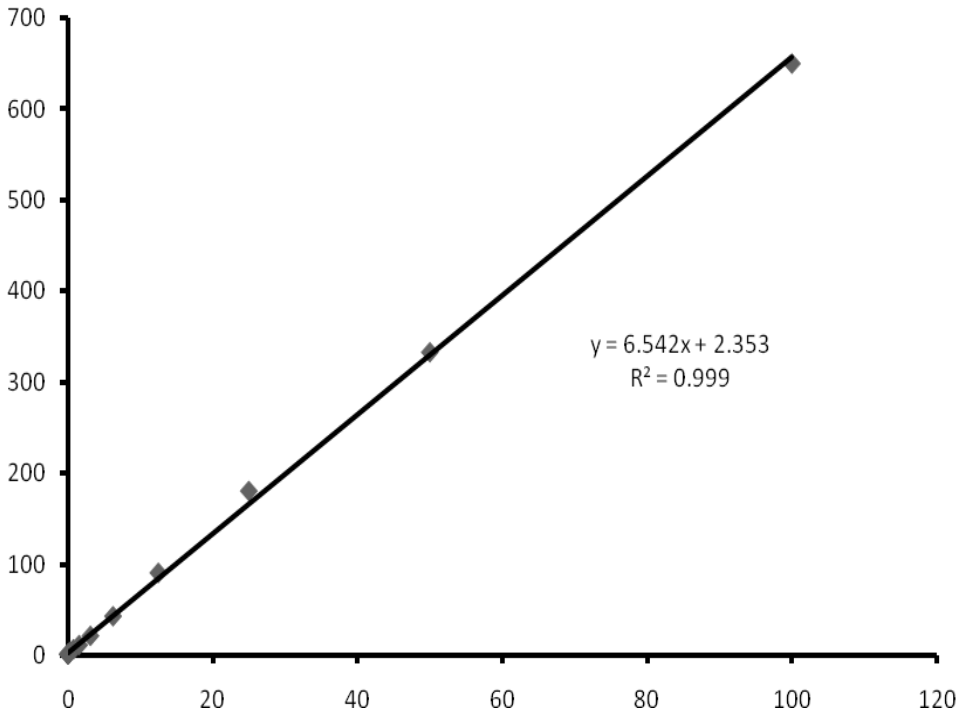

Figure 2. Standard curve for nitrite.

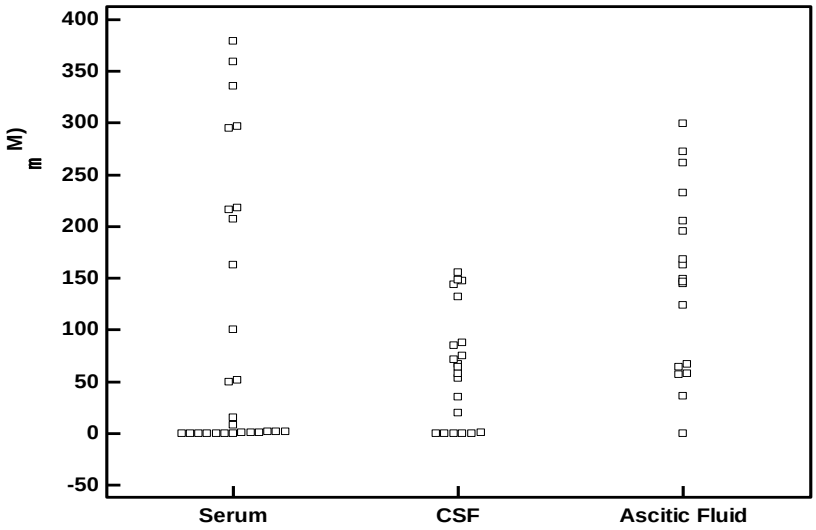

Figure 3. Nitrite distribution in various biological fluids. Plotting of the data from the earlier tables is done for distribution. Nitrite levels in the three fluids have been found to be zero in several samples.

Table 1. Data regarding standard curve of nitrite.

Serial No.	Conc. (µg/mL)	Conc. (µM)	Height (mV)
1	0.012	0.26	0.21
2	0.024	0.52	0.43
3	0.048	1.04	0.83
4	0.098	2.33	1.04
5	0.195	4.24	2.37
6	0.391	9.5	3.13
7	0.781	16.98	5.89
8	1.562	33.96	10.61
9	3.125	67.93	20.69
10	6.25	135.87	42.28
11	12.5	200.2	90.06
12	25	543.48	180.13
13	50	1086.96	332.86
14	100	2173.91	650.74

Table 2. Standard curve parameters for nitrite.

Sample	Slope	Intercept	r-square
Nitrite	6.542	2.352	0.999

Table 3. Data of between-batches precision and accuracy for nitrite levels in three batches of independent experiments.

Batch No.	Low Conc.(LC) µg/mL	Medium Conc. (MC)µg/mL	High Conc. (HC)µg/mL
Ntrite-01	3.18	25.66	99.11
	3.27	24.91	98.64
	3.17	26.45	99.27
Nitrite-02	2.99	24.74	98.83
	3.08	25.68	99.66
	3.12	24.98	99.54

Nitrite-03	3.02	25.21	99.38
	3.1	25.83	99.05
	3.15	25.44	99.08
Mean	3.12	25.43	99.17
SD	0.09	0.54	0.33
N	9.00	9.00	9.00
Nominal	3.13	25.00	100.00
% CV	2.74	2.11	0.33
% accuracy	99.68	101.73	99.17

Table 4. Data of within-batch precision and accuracy for nitrite levels in a batch (one experiment).

Batch (nitrate)	**Low Conc. µg/mL (LC)**	**Medium Conc. µg/mL (MC)**	**High Conc. µg/mL (HC)**
01	2.98	25.66	99.11
02	3.17	24.91	98.64
03	3.07	26.45	99.27
04	2.99	24.74	98.83
05	3.05	25.68	99.66
06	3.12	24.98	99.54
Mean	3.063	25.40	99.18
SD	0.074	0.65	0.39
N	6	6	6
Nominal	3.13	25	100
%CV	2.41	2.55	0.40
%Accuracy	97.87	101.61	99.18

Table 5. Parameters regarding the HPLC analysis of nitrite.

Parameter	**Result**
Mobile phase	Water at pH 6.4
Retention Time	3.5 ± 0.1 min
LOD	0.006 µg/mL
LOQ	0.012 µg/mL
Wavelength	220nm

Table 6. Data for recovery of nitrite in the serum. The percent recovery of nitrite from spiked serum was measured as given the methods section. Data is given below.

Conc. Spiked (µg/ml)	**Peak height of used conc.**	**Height of conc. In the sample**	**Recovery from Sample (210)**	**Percentage recovery**
50	329.36	320.30	48.60	97.20
25	160	190.62	28.78	115.11
5	35.02	40.04	5.76	115.22

Table 7. Changes in nitrite levels in sera of patients. Blood was collected from the hospital. Serum was collected from blood. Serum (195 µL) was mixed with 5 µL stock solution of sodium nitrite (1 mg/mL) to give final concentration of 25 µg/mL nitrite. This solution was injected into 20 µL sample loop in HPLC. Data was calculated by subtracting the added nitrate in the serum which is given below in the far-right columns in µg/mL and µM conc. in the Table.

Serial No.	**Serum code**	**Height (mV)**	**Height of spiked nitrite (25µg/mL)**	**Height of serum**	**Conc. of (diluted sera) (µg/mL)**	**Actual conc. (µg/mL)**	**Nitrite (µM)**
1	86	168.03	165.90	3.13	0.12	0.36	7.73
2	90	169.81	165.90	3.91	0.24	0.72	15.57
3	214	173.20	165.90	7.30	0.76	2.27	49.37
4	210	173.44	165.90	7.54	0.79	2.38	51.75
5	216	167.35	165.90	2.45	0.02	0.05	1.02
6	223	184.55	165.90	18.64	2.49	7.47	162.44
7	229	92.83	165.90	0.00	0.00	0.00	0.00
8	242	168.20	165.90	0.30	-0.01	-0.02	-0.53
9	200	189.01	165.90	23.11	3.17	9.52	206.93
10	239	190.12	165.90	24.22	3.34	10.03	218.01
11	218	189.90	165.90	24.00	3.31	9.93	215.81
12	221	198.03	165.90	32.13	4.55	13.65	296.83
13	222	206.31	165.90	40.40	5.82	17.45	379.36
14	230	149.77	165.90	0.00	0.00	0.00	0.00
15	267	201.89	165.90	35.98	5.14	15.42	335.29
16	206	124.75	165.90	0.00	0.00	0.00	0.00
17	237	164.24	165.90	0.00	0.00	0.00	0.00
18	226	135.71	165.90	0.00	0.00	0.00	0.00
19	227	204.30	165.90	38.40	5.51	16.53	359.40
20	211	197.89	165.90	31.98	4.53	13.59	295.41
Sum **Mean** **N** **Max** **Min** **SD**							**2594.92** **129.72** **20** **379.36** **0** **143.83**

Table 8. Data for recovery of nitrite in CSF. The percent recovery of nitrite from spiked CSF was measured as given the methods section. Data is given below.

Conc. Spiked (µg/mL)	**Peak height of used conc.**	**Height of conc. In the sample**	**Recovery from Sample (SAJI)**	**Percentage recovery**
50	329.36	305.87	46.40	92.79
25	160	170.76	25.74	102.97
5	35.02	40.88	5.89	117.79

Table 9. Changes in nitrite levels in CSF of patients. CSF was collected from various wards of BV Hospital. It was centrifuged and the supernatant was diluted after spiking with 20 µg/mL nitrite and injected into HPLC. Final concentration was calculated by subtracting the added nitrite.

Serial No.	CSF code	Height (mV)	Height of spiked nitrite	Height of nitrite	Conc. of (diluted CSF) (µg/mL)	Actual conc. (µg/mL)	Nitrite in CSF (µM)
1	SHO	94.30	133.19	0	0	0	0
2	KA	133.57	133.19	5.38	0.46	0.93	20.13
3	F.SR	163.49	133.19	10.30	1.21	2.43	52.84
4	AASIA	105.98	133.19	0	0	0	0
5	AAYIA	118.22	133.19	0	0	0	0
6	AZ(86)	157.19	133.19	23.99	3.31	6.62	143.86
7	AAN	135.71	133.19	2.514	0.02	0.05	1.08
8	AD	198.75	133.19	15.56	2.02	4.04	87.79
9	ALI	155.41	133.19	22.22	3.04	6.07	132.05
10	ASIF	115.23	133.19	0	0	0	0
11	FZN	157.90	133.19	24.71	3.42	6.83	148.58
12	MSTM	248.27	133.19	15.08	1.95	3.90	84.60
13	MUZA.	146.30	133.19	13.11	1.64	3.29	71.48
14	SAJI	140.81	133.19	7.62	0.81	1.61	35.02
15	SAMIA	286.76	133.19	13.57	1.71	3.40	74.54
16	SH	187.78	133.19	24.58	3.40	6.80	147.75
17	SHAN	131.57	133.19	0	0	0	0
18	SUH	279.01	133.19	25.82	3.59	7.17	155.96
19	ZAIN.	145.61	133.19	12.42	1.54	3.07	66.89
20	WASM	164.24	133.19	11.05	1.33	2.66	57.81
Sum							**1280.38**
Mean							**64.01**
N							**20**
Max							**155.96**
Min							**0**
SD							**57.19**

Table 10. Data for recovery of nitrite in ascetic fluid. The percent recovery of nitrite from spiked ascitic fluid was measured as given the methods section. Data is given below.

Conc. Spiked (µg/mL)	Peak height of used conc.	Height of conc. In the sample	Recovery from Sample (HFZ)	Percentage recovery
50	329.36	375.69	57.07	114.14
25	160	145.77	21.92	87.69
5	35.02	32.564	4.62	92.36

Table 11. Changes in nitrite levels in ascetic fluid of patients. Ascitic fluid of liver cirrhotic patients was collected from the BV Hospital. It was centrifuged and after suitable dilution, it was spiked with nitrite to a final concentration of 40 µg/mL before injection into HPLC.

Serial No.	Ascitic fluid code	Height (mV)	Height of spiked (20 µg/mL)	Height of ascitic fluid	Conc. of (diluted ascitic fluid) (µg/mL)	Actual conc. (µg/mL)	Nitrite conc. (µM)
1	Zubaida	145.17	133.19	11.98	1.47	2.94	63.95
2	Nawab	115.23	133.19	0.00	0.00	0.00	0
3	A2	260.82	133.19	27.63	3.86	7.73	167.98
4	Aa	158.01	133.19	24.82	3.43	6.87	149.33
5	Aayia	154.20	133.19	21.00	2.85	5.70	123.95
6	Aba	144.14	133.19	10.95	1.31	2.63	57.15
7	Abbas	186.39	133.19	33.20	4.72	9.43	205.01
8	Zubair	164.96	133.19	31.77	4.50	8.99	195.53
9	Ilyas	157.27	133.19	24.07	3.32	6.64	144.36

10	Nawaz	244.19	133.19	11.00	1.32	2.64	57.54
11	Ishf.	160.01	133.19	26.81	3.74	7.48	162.57
12	ALS	145.61	133.19	12.42	1.54	3.08	66.89
13	HFZ.	140.95	133.19	7.76	0.83	1.65	35.95
14	Faiz	170.57	133.19	37.38	5.35	10.71	232.79
15	Mum.	180.54	133.19	47.35	6.88	13.76	221.88
16	NB	174.85	133.19	41.66	6.01	12.02	193.83
17	GA	135.39	133.19	2.20	0.00	0.00	0
18	EB.	151.53	133.19	0.00	0.00	0.00	0
19	JM	120.43	133.19	0.00	0.00	0.00	0
20	PA	176.46	133.19	43.27	6.25	12.51	201.75
Sum							
Mean							**2495.14**
N							**146.77**
Max							**17**
Min							**299.05**
SD							**0**
							89.94

Conclusions

A useful mediator of physiological and pathological processes is nitric oxide. It is produced in a variety of tissues and cells and functions as a neurotransmitter, vasodilator, and killer molecule in the immune system. The arginine–nitric oxide pathway may produce nitrite, a metabolic product of nitric oxide, but it would be undetectable due to its quick oxidation to nitrate. One possible illustration of nitrite formation through this pathway is the methemoglobinemia observed in infants with diarrhea. It was developed and confirmed that the ascitic fluid, CSF, and serum could all contain nitrite using the straightforward and repeatable HPLC method. The developed method was extremely basic, using a C18 column as the stationary phase and distilled water as the mobile phase. The available data indicates that this technique can be used to accurately determine the nitrite concentrations in these fluids.

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