Determination of nitrate in biological fluids by HPLC

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Abstract: Nitrate is the stable product of nitric oxide, which is physiologically active radical, an immunomodulator and a neuromodulator; its quantification in biological fluids is important to study the physiological and biochemical nature. Therefore, the purpose of this study was to quantify nitrate in different biological fluids like serum, cerebrospinal fluid (CSF) and ascetic fluid (ASF) using HPLC technique. A new HPLC method for the estimation of nitrate in serum, CSF and ASF was developed using the mobile phase of 1.0mM each of Na_2CO_3 and $NaHCO_3$ (1:1, v/v, pH 5 with H_3PO_4) at a flow rate of 1.0mLmin^{-1}. Eluate was detected at 220nm with the retention time of nitrate 2.55 min. The LOD and LOQ values of nitrate were 0.03µgmL^{-1} and 0.098µgmL^{-1}, respectively. Nitrate was eluted through SAX Hypersil column of 150 × 4.6mm, id, 5µm particle size. Run time was 10min. The method was validated according to the FDA guidelines and was found linear in the range of 0.39 to 50µgmL^{-1} and CV was <3%, within limits of FDA guidelines. The method was used successfully for the estimation of nitrate in biological fluids like serum, CSF and ASF of 20 patients each. This is an alternate and reproducible method for the detection of nitrates in biological fluids.

Keywords: Ascitic fluid, biological fluids, cerebrospinal fluid, HPLC, nitrate, serum.

INTRODUCTION

Nitric oxide (NO) is an important mediator of both physiological and pathological processes. It is not only a vasodilator and neurotransmitter but also an immunomodulator (Palmer *et al*., 1988; Kolb and Kolb, 1992). Nitric oxide has short half-life, *in vivo*, of about 0.1 second (Kelm and Schrader, 1990), after which it is metabolized to stable products, that is, nitrite and nitrate in plasma or other physiological fluids where it remains stable for several hours (Grube and Kelm, 1994).

Nitrite, nitrate, nitroso compounds and other nitrogen oxide containing compounds lead to the formation of reactive free radical, nitric oxide, which activate guanylyl cyclase, increase the cellular levels of cyclic-GMP which activates PKG (cGMP-dependent protein kinase G) and modulates the activities of cyclic nucleotide phosphodiesterases (PDEs 2, 3 and 5) in a range of cell types. In smooth muscle, PKG catalyzes the phosphorylation of various proteins by this mechanism. Another important target of this kinase is myosin light chain phosphatase, which is activated on binding cGMP dependent protein kinase and leads to dephosphorylation of the light chain myosin chain and therefore promoting vasorelaxation (Waldman and Murad 1987).

After low intake of nitrate and nitrite, an excess in nitrate excretion is observed which indicates its endogenous synthesis and it corresponds to 62mg of nitrate per day. In gastrointestinal infections, the excretion of nitrate greatly increases as a result of increased endogenous (non-bacterial) nitrate synthesis (Wishnok *et al*., 1995) through the arginine-nitric oxide pathway involving nitric oxide synthesis (Leaf *et al*., 1989). It is known that nitrate is reduced to nitrite by bacterial and mammalian metabolic pathways via the nitrate reductase (Gangolli *et al*., 1994). Individuals having gastrointestinal disorders and achlorhydria can have high levels of nitrite, which may be up to 6mgL^{-1} (Dolby *et al*., 1984).

These studies reflect the significance of the determination of nitrate in biological fluids like serum, cerebrospinal fluid (CSF), ascetic fluid (ASF) and others. Quantitative determination of nitrate has been carried out by GC-MS (Tsikas and Gutzki, 1997; Tsikas *et al*., 1999; Tsikas, 2000), capillary ion electrophoresis (Trushina *et al*., 1997) and quantitative analysis in human biological fluids (Tsikas, 2005; Tsikas, 2007). Troska *et al.,* (2013) determined nitrite and nitrate using isotachophoresis with capillary electrophoresis on a poly-(methylmethacrylate) chip using conductivity detector. Masar *et al.,* (2015) used microchip capillary electrophoresis using solid-phase micro-extraction prior to an online combination of isotachophoresis with capillary zone electrophoresis. The method was sensitive with 3-9nM detection of nitrite and nitrate in diluted CSF samples Nitrate and nitrite has also been determined by colorimetric micro fluidic paper-based analytical device with immobilized zinc particles (Jayawardane *et al.,* 2014), by bienzymatic biosensors using super oxide dismutase and nitrate reductase coimmobilized on carbon nanotubes coated with cellulose acetate membrane (Madasamy *et al.,* 2014) and capillary zone electrophoresis employing borate buffer without deproteinization of CSF samples (Zunic *et al.,* 1999).

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These methods require specialized equipment, which is not available in every laboratory. However, ion exchange method (Murad and Korenaga, 2000) and HPLC methods (Jedlickova *et al.*, 2002; Tahboub, 2008; Angou *et al.*, 2013) are published. A Cd-Zn reduction method for the determination of nitrate in blood has previously been reported by our group (Ashraf *et al.*, 2006). The present method is an alternate and validated HPLC method for the estimation of nitrate in biological fluids like serum, CSF and ASF.

MATERIALS AND METHODS

Materials

Sodium nitrate, HPLC grade methanol and acetonitrile were bought from Merck, Germany. Sodium nitrite was purchased from Fluka, Switzerland. Hydrochloric acid, sodium chloride, phosphoric acid, de-ionized double distilled water were of HPLC grade.

Method development

An HPLC method was developed, standardized for the analysis of nitrate in serum, CSF and ASF. In the development process, various systems of mobile phase with several combinations were tested. Finally, 1.0mM Na_2CO_3: 1.0mM $NaHCO_3$ (50:50, v/v) at pH 5 was found to be the most suitable at a flow rate of 1.0 $mLmin^{-1}$ Nitrate was eluted through SAX Hypersil column of 150 × 4.6mm, id, with 5µm particle size. Run time of every elution was 10 min, which was detected at 220 nm. LOD was0.03μgmL^{-1} and LOQ was 0.098μgmL^{-1}. The method was found linear in the range 0.39μgmL^{-1} to 50μgmL^{-1}.

Preparation of mobile phase

Mobile phase was made by mixing 1mM Na_2CO_3 and 1M $NaHCO_3$ at a ratio of (50:50) and contents filtered through 0.45µm membrane filters and pH 5was adjusted using orthophosphoric acid. Mobile phase was used after degassing.

Preparation of stock solutions

Stock solution of nitrate (1.0$mgmL^{-1}$) was constituted in double distilled water. Further dilutions were made up to 0.098μgmL^{-1}. Fresh solution was prepared daily, filtered and degassed by sonication.

Peak identification and retention times

The peak of nitrate was identified and confirmed by the increase or decrease in size of the peak with change of concentration of standard solution. During application of this method, retention time of nitrate was 2.55 min. A representative chromatogram is given in fig. 1.

Treatment of biological samples

The study protocols and procedures were approved by the Ethics Committee (a sub-committee of Board of Studies of the Department) of the Islamia University of Bahawalpur regarding use of biological fluids in this study. Blood (serum), CSF, ASF were collected from the Biochemistry Laboratory of the Quaid-i-Azam Medical College as soon as these samples of admitted patients were received from the hospital indoor wards for analyses and immediately placed at -70^oC.

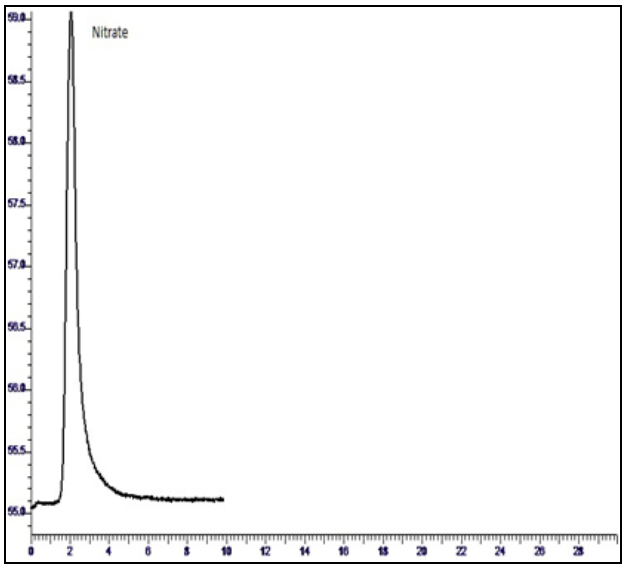

Fig. 1: A representative chromatogram of nitrate elution profile.

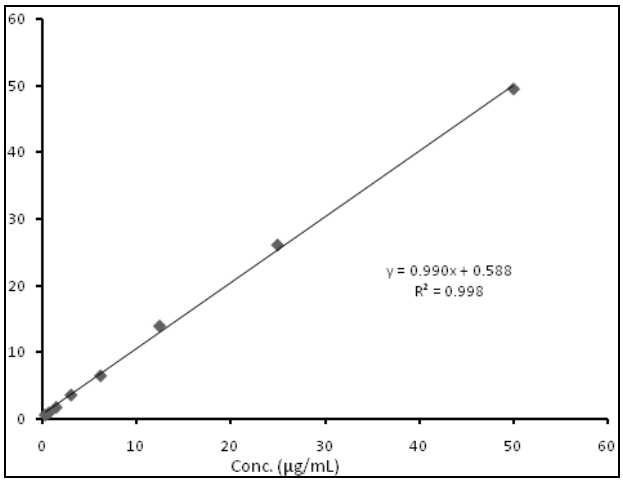

Fig. 2: Standard curve for nitrate at 220 nm.

Serum

Serum (200µL) was taken in 1.5mL tubes followed by the addition of 400µL HPLC grade water. Contents were vortex mixed for 5 min and 20µL saturated solution of $ZnSO_4$ was added. Contents were centrifuged for 10 min at 3000 rpm and supernatant was taken as a source of nitrate. In other experiments, 195µL of this supernatant, 5 µL of stock solution of nitrate was added (25μgmL^{-1}) during method development as an external source to maintain levels to quantifiable limits and reduce the error of quantification. This was especially more important in those samples where nitrate was in negligible and undetectable limits.

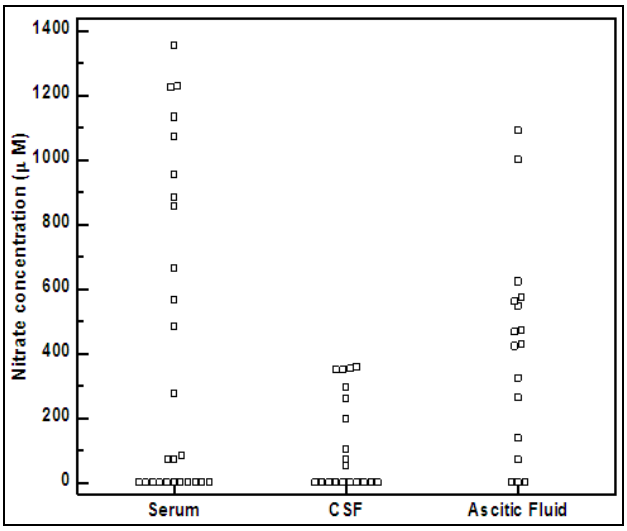

Fig. 3: Distribution of nitrate among different biological fluids. Data in tables 7-9 is plotted here for frequency distribution. Many samples have shown zero levels of nitrate in the three biological fluids as shown in this graph.

Cerebrospinal fluid

CSF (200µL) was treated as with the serum except that 240µL of supernatant, 10µL (40µgmL^{-1}) of stock solution (as an external source) were mixed during method development

Ascetic fluid

ASF (200µL) was treated as mentioned for serum and CSF except that it was mixed 600µL HPLC grade water. Finally, 240µL of this mix was taken and 10 µL of stock solution of nitrate was added (40µg mL^{-1}) as for CSF as an external source.

Validation of HPLC method for the determination of nitrate

The method was validated step by step according to standard analytical practice guidelines.

Linearity and range

Linearity of the analytical method is its ability to obtain test results within a given range and is directly proportional to the concentration of the sample. It was determined by plotting calibration curve to find out the relationship between instrumental response and known concentrations of pure nitrate samples. The standard stock solution was diluted with mobile phase to prepare a set of solutions of different concentrations ranging from 0.39 to 50µgmL^{-1}. The calibration curve of nitrate (99.5% purity) was prepared in the mobile phase. Each sample was run in triplicates and data was plotted to calculate the parameters of calibration curve (fig. 2). The values of slope, intercept and r-square for nitrate were 0.99, 0.588 and 0.998, respectively, as given. The values of these parameters were found to be reliable with FDA guidelines of method validation.

Accuracy

Accuracy of an analytical method depicts the closeness of test results with the mean value. It was determined by selecting low, medium and high concentrations (1.56, 12.5, 50.00µgmL^{-1}) of nitrate in triplicate. The mean values (low, medium and high) of accuracy for pure nitrate were 99.29, 102.54 and 99.75%, respectively (table 1).

Precision

Precision of the method was performed by measuring elution profile of nitrate at three different concentrations (low, medium and high) in a range in triplicate. This assay was repeated for three different batches. The precision was found within the limits of FDA guidelines (tables 1-2).

Sensitivity

The limit of detection (LOD) and limit of quantification (LOQ) values of nitrate were 0.03 and 0.098µgmL^{-1}, respectively (Table 3) and therefore the method was found highly sensitive.

Robustness

The method was found robust under varied conditions of flow rate (±0.05mLmin^{-1}), mobile phase compositions (±0.5 pH variation) and wavelength (±6-8 nm) and no significant changes were observed in the elution profiles (data not shown). Therefore, method was found robust within limits as after minor changes in conditions.

Application of method

This developed method was applied in the determination of nitrate levels in serum, CSF and ASF. These results are given in Tables 4, 5 and 6 that show its wider applications in the quantification of nitrate in biological fluids.

RESULTS

The objectives of the study were the development and validation of an analytical method for the determination of nitrate in different biological fluids. Standard curves were constructed and assayed in triplicates to evaluate linearity, accuracy, precision, sensitivity (LOD and LOQ) and stability of method.

During the method developmental process, mobile phase of different compositions with several combinations of organic and aqueous phase were tested. Final selected mobile phase for nitrate determination was 1.0mM Na_2CO_3 and 1.0mM $NaHCO_3$ in the ratio of 50:50 (% v/v) at pH 5. After much experimentation, the flow rate was adjusted to 1.0mLmin^{-1}. The retention time for nitrate was 2.55 min. The standardization and validation of analytic method was carried out by the parameters, *i.e.* linearity, accuracy, precision, stability, sensitivity and robustness as given in results section. The method was successfully applied for the determination of nitrate in biological fluids and was found to be simple, sensitive and stable.

Table 1: Data of between-batches precision and accuracy for nitrate levels in three batches of independent experiments

Batch No.	Low Conc. µgmL^{-1} (LC)	Medium Conc. µgmL^{-1} (MC)	High Conc. µgmL^{-1} (HC)
Nitrate-01	1.58	13.54	49.41
	1.56	12.59	50.68
	1.61	12.27	49.85
Nitrate-02	1.53	12.94	50.14
	1.48	13.07	50.29
	1.52	12.73	49.23
Nitrate-03	1.56	12.46	48.97
	1.59	12.79	49.87
	1.51	12.97	50.45
Mean	1.55	12.82	49.88
SD	0.042	0.37	0.58
N	9	9	9
Nominal	1.56	12.50	50.00
% CV	2.71	2.91	1.16
% Accuracy	99.29	102.54	99.75

Table 2: Data of with-in-batch precision and accuracy for nitrate levels in a batch (one experiment)

Batch (nitrate)	Low Conc. µgmL^{-1} (LC)	Medium Conc. µgmL^{-1} (MC)	High Conc. µgmL^{-1} (HC)
01	1.58	13.14	49.41
02	1.56	12.59	50.68
03	1.61	12.67	49.85
04	1.53	12.94	50.14
05	1.48	13.07	50.29
06	1.52	12.73	49.23
Mean	1.55	12.86	49.93
SD	0.046	0.23	0.55
N	6	6	6
Nominal	1.56	12.5	50
%CV	2.99	1.76	1.01
%Accuracy	99.15	102.85	99.87

Table 3: Parameters regarding the HPLC analysis of nitrate

Parameter	Result
Mobile phase	1.0mM Na_2CO_3: 1.0mM $NaHCO_3$
Flow Rate	1.0mLmin^{-1}
Retention Time	2.55±0.1min
LOD	0.03µgmL^{-1}
LOQ	0.098µgmL^{-1}
Wavelength	220nm

Table 4: Data for recovery of nitrate in serum. The percent recovery of nitrate from spiked serum was measured as given the methods section. Data is given below. Data is mean of 3 independent experiments and error is <5%

Conc. of spiked samples (µgmL^{-1})	Peak height of used conc.	Height of conc. in the sample	Recovery from sample	Percentage recovery
50	50.09	51.26	51.11	102.22
25	25.33	24.58	24.19	96.76
5	4.86	4.96	4.82	96.40

Table 5: Data for recovery of nitrate in CSF. The percent recovery of nitrate from spiked CSF was measured as given the methods section. Data is given below.

Conc. of spiked nitrate (μgmL^{-1})	Peak height of used conc.	Height of conc. In the sample	Recovery from Sample (ALI)	Percentage recovery
50	50.02	49.19	49.48	98.96
25	25.13	25.24	25.83	103.32
5	4.86	4.78	4.92	98.40

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

Conc. Spiked (μgmL^{-1})	Peak height of used conc.	Height of conc. In the sample	Recovery from Sample	Percentage recovery
50	50.09	50.04	50.03	100.60
25	24.06	24.03	24.05	96.20
5	4.86	4.83	4.88	97.60

Table 7: Changes in nitrate levels in sera of patients. Data was calculated by subtracting the added nitrate in the serum which is given below in the far right columns in μgmL^{-1} and μM conc. in the Table. Details are given in the text

Serial No.	Serum code	Height (mV)	Height of spiked nitrate (50μgmL^{-1})	Height of serum peak	Conc. of nitrate (diluted serum) (μgmL^{-1})	Actual conc. (μgmL^{-1})	Nitrate in Serum (μM)
1	86	63.25	49.50	13.75	13.30	66.49	1072.42
2	90	58.25	49.50	8.75	8.25	41.23	664.96
3	214	66.70	49.50	17.20	16.78	83.91	1353.37
4	210	55.74	49.50	6.24	6.01	30.04	484.44
5	216	63.99	49.50	14.49	14.04	70.22	1132.62
6	223	60.67	49.50	11.17	10.97	54.87	885.00
7	229	61.54	49.50	12.04	11.83	59.16	954.19
8	242	65.18	49.50	15.68	15.24	76.21	1229.23
9	200	65.13	49.50	15.63	15.19	75.97	1225.32
10	239	50.97	49.50	1.47	0.89	4.45	71.85
11	218	57.02	49.50	7.52	7.01	35.04	565.09
12	221	50.97	49.50	1.47	0.89	4.45	71.85
13	222	31.47	49.50	18.04	0.00	0.00	0.00
14	230	30.55	49.50	18.95	0.00	0.00	0.00
15	267	60.60	49.50	11.10	10.62	53.09	856.31
16	206	49.66	49.50	0.16	0.00	0.00	0.00
17	237	43.59	49.50	-5.91	0.00	0.00	0.00
18	226	53.47	49.50	3.97	3.42	17.11	275.90
19	227	46.16	49.50	-3.34	0.00	0.00	0.00
20	211	47.36	49.50	-2.14	0.00	0.00	0.00
Sum Mean Max N Min SD							10923.44 542.13 1353.37 20 0 508.27

Table 8: Changes in nitrate levels in CSF of patients. CSF was collected, centrifuged and the supernatant was diluted after spiking with 40µgmL^{-1} nitrate and injected into HPLC. Final concentration of nitrate was calculated by subtracting the added nitrate. Details are given in the text

Serial No.	CSF code	Height (mV)	Height of spiked nitrate (mV)	Height of CSF nitrate (mV)	Conc. of nitrate (diluted CSF) (µgmL^{-1})	Actual nitrate conc. (µgmL^{-1})	Nitrate in CSF (µM)
1	SHO	42.28	40.19	2.09	1.52	4.56	73.51
2	KA	46.84	40.19	6.65	6.12	18.37	296.24
3	F.SR	36.11	40.19	-4.07	0.00	0.00	0.00
4	AASIA	48.00	40.19	7.81	7.30	21.89	353.13
5	AAYIA	44.80	40.19	4.61	4.07	12.20	196.82
6	AZ(86)	39.53	40.19	-0.66	0.00	0.00	0.00
7	AAN	39.08	40.19	-1.11	0.00	0.00	0.00
8	AD	40.31	40.19	0.12	0.00	0.00	0.00
9	ALI	42.91	40.19	2.72	2.16	6.47	104.30
10	ASIF	39.38	40.19	-0.81	0.00	0.00	0.00
11	FZN	47.95	40.19	7.76	7.24	21.73	350.54
12	MSTM	37.92	40.19	-2.27	0.00	0.00	0.00
13	MUZA.	37.52	40.19	-2.67	0.00	0.00	0.00
14	SAJI	41.87	40.19	1.68	1.10	3.31	53.37
15	SAMIA	45.73	40.19	5.54	5.37	16.12	259.94
16	SH	39.17	40.19	-1.02	0.00	0.00	0.00
17	SHAN	37.36	40.19	-2.83	0.00	0.00	0.00
18	SUH	47.92	40.19	7.73	7.22	21.65	349.17
19	ZAIN.	48.10	40.19	7.91	7.39	22.18	357.82
20	WASM	37.05	40.19	-3.14	0.00	0.00	0.00
Sum							2394.84
Mean							119.74
N							20
Max							357.82
Min							0
SD							149.37

DISCUSSION

Many methods have been developed for the individual or simultaneous determination of nitrate and nitrite and documented separately in various biological fluids. Murad and Korenaga (2000) described an HPLC method for the determination of nitrate and nitrite in saliva. They used 2.7mM Na_2CO_3 and 0.3mM $NaHCO_3$. The column used was Ion Pac AS12A analytical column (200 x 4). Flow rate was 1.5mLmin^{-1} and detected by conductivity detector. LOD and LOQ values were 15.0µgL^{-1} and 33.5 µgL^{-1}, respectively.

Tahboub (2008) reported another method for the estimation of nitrate (along with nitrite) in cell culture medium. The principle of this method was pre-column derivatization of nitrite under acidic conditions with 2,3-diaminonaphthalene to yield 2,3-naphthotriazole. Nitrate was reduced into nitrite by nitrate reductase before the derivatization step. A 5 µm C_{18} column (250 x 4.6 mm, id) was used for the separation of 2,3-naphthotriazole. The mobile phase of 15mM phosphate buffer (pH 7.5) containing 35% acetonitrile was used at a flow rate 1.0 mL min^{-1}. Absorbance was noticed at 369 nm and fluorescence measured at 375nm (excitation) and 415nm (emission). Linearity of this method was in the range of 0.03-2.0µM for fluorescence measurement and 0.5-50µM for UV-visible range.

In the present study, nitrate was directly determined in serum, CSF, ASF with this sensitive and validated HPLC method. The method was found linear in the range from 0.39 to 50µgmL^{-1}. The values of slope, intercept and r-square for nitrates were 0.99, 0.588 and 0.998, respectively. The mean values (low, medium and high) of accuracy for nitrates were 99.29, 102.54 and 99.75%, respectively. The CV with-in-batch for low, medium and high concentrations of nitrates were 2.99, 1.76 1.01% and between-batches were 2.71, 2.91 and 1.16%, respectively. The LOD and LOQ values of nitrates were 0.03 and 0.098 µgmL^{-1}, respectively. The retention time of nitrate was 2.55 min.

Table 9: Changes in nitrate levels in ascetic fluid (ASF) of patients. ASF of liver cirrhotic patients was collected, centrifuged, diluted, and spiked with nitrate to a final concentration of 40µgmL^{-1} before injection into HPLC. Final concentration of nitrate was calculated by subtracting the added nitrate. Details are given in the text.

Serial No.	ASF code	Height (mV)	Height of spiked nitrate (mV)	Height of ASF nitrate (mV)	Conc. of nitrate (diluted ASF) (µgmL^{-1})	Actual conc.(µgmL^{-1})	Nitrates in ASF (µM)
1	ZUB	47.28	40.19	7.09	6.57	26.28	423.85
2	NAWA	36.84	40.19	0.00	0.00	0	0.00
3	A2	56.11	40.19	15.93	15.49	61.98	999.61
4	AA	48.00	40.19	7.81	7.30	29.19	470.84
5	AAYIA	44.80	40.19	4.61	4.07	16.27	262.43
6	ABA	49.53	40.19	9.34	8.84	35.37	570.48
7	ABBAS	39.08	40.19	0.00	0.00	0.00	0.00
8	ZUBAIR	50.31	40.19	10.12	9.63	38.52	621.31
9	ILYAS	42.91	40.19	2.72	2.16	8.62	139.07
10	NAWAZ	49.38	40.19	9.19	8.69	34.76	560.64
11	ISHFAQ	47.95	40.19	7.76	7.24	28.98	467.38
12	ALS	37.92	40.19	0.00	0.00	0.00	0.00
13	HFZ	57.52	40.19	17.33	16.91	67.64	1091.04
14	FAIZ	41.87	40.19	1.68	1.10	4.41	71.16
15	MUMT	45.73	40.19	5.54	5.01	20.03	323.04
16	NB	49.17	40.19	8.98	8.48	33.92	547.02
17	GA	47.36	40.19	7.17	6.65	26.59	428.93
18	EB	37.92	40.19	0.00	0.00	0.00	0.00
19	JM	38.10	40.19	0.00	0.00	0.00	0.00
20	PA	37.05	40.19	0.00	0.00	0.00	0.00
Sum							6976.8
Mean							436.05
N							20
Max							1091.04
Min							0
SD							313.45

Application of method in analysis of biological fluids

The newly developed method was successfully applied to the determination of nitrates in sera, CSF and ASF. Data of individual patients is given in tables 7-9 and plotted as fig 3. Serum nitrate levels were 542.13±508.27µM (mean ±SD, n=20) with minimum and maximum levels of undetectable concentration (zero) and 1353.37µM. Sera of six patients showed undetectable levels of nitrate though the nature of the disease of the patients was not known (table 7). Variations in nitrate levels are given in table 8. Data shows that 50% of the CSF of patients tested (n=10) had again undetectable levels of nitrate and rest of 10 patients exhibited nitrate levels up to 357.82 µM. CSF nitrate levels were 119.74±149.37 µM (mean ±SD, n=20) with minimum and maximum levels of zero and 357.82 µM (table 8). Nitrate levels in ASF of cirrhotic patients showed six patients with undetectable levels and rest of 14 patients with maximum levels of 1091.04 µM. ASF nitrate levels were 436.05±313.45 µM (mean ±SD, n=20). The spread of the variations in nitrate levels is depicted in fig 3. The data is from diseased patients without knowing details of their disease and therefore cannot be compared with the reported data. Nonetheless, the method has successfully resulted in the detection of nitrate from minimum to maximum levels in all the three studied fluids.

CONCLUSIONS

An HPLC method was developed and validated for the estimation of nitrate in serum, CSF and ASF. During the study, CV was<3% that showed reproducibility of all parameters including linearity & range, accuracy, precision, specificity, etc. The method can be applied to various biological fluids.

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