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DETERMINATION OF NUCLEOSIDES IN SERUM BY LC-MS/MS FOLLOWING PROTEIN PRECIPITATION WITH ORGANIC SOLVENT

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Abstract- A lot of studies have been used to quantify the nucleosides in sick individuals. A liquid chromatography mass spectrometry (LC–MS) method was developed and validated for the determination of nine nucleosides in blood serum. The nucleosides were analyzed after a simple protein precipitation using iced organic solvents (acetone). The method was linear for all analytes with correlation coefficients (r) between 0.9872 and 0.9997. The lower limit of quantification (LLOQ) was 0,0050 µg/L e 1,000 µg/L with acceptable validation parameters. The recovery of nucleosides was assessed by extraction and showed good results, demonstrating that cleaning procedure by protein precipitation is fast, reliable and demand small quantities of organic solvent. This methodology can be used to evaluate the expression of nucleosides as biomarkers for different diseases including malignancies. Moreover, simultaneous determination of nucleosides in a single run and quick clean up process by protein precipitation by organic solvents makes it compatible with routine analysis in clinical diagnostics laboratories.

Keywords **-**Nucleosides, cancer, tumor biomarkers, blood serum**.**

I. INTRODUCTION

Nucleosides and their metabolites play an important role in a variety of fundamental biological processes are excreted in human biological fluids, such as urine and blood, during normal cell activity.In the post-transcpitional stage of transfer ribonucleic acid (tRNA), the four common nucleosides (i.e. adenosine, guanosine, cytidine and uridine) are released inside the cells and are further reincorporated in nucleic acids or degraded by the action of specific enzymes. However, if the nucleosides are modified by the action of some other enzyme or the individual has a disease state, they can not be reused, being completely excreted in urine or bloodstream.[1,2]

In the 60s, it is known that the nucleoside originated in tRNA in order to increase their efficiency in various biological processes. In the same decade they have been reported on the various concentrations of nucleic acids in individuals with cancer by quantification of pseudouridine.[3]Soon after, some studies have shed light on the two main reasons for the increased excretion of these nucleosides: High activity of methyltransferase enzymes in tumor cells and the higher speed recycling of tRNA molecules in tumor cells relative to healthy cells.[4]

From these findings, several studies have been used to quantify the nucleosides in sick individuals. Since studies have been conducted evaluating the concentration of nucleosides in biological fluids (urine and blood serum) of healthy individuals and different types of malignancies such as breast cancer[5,6],Bladder[7], colorectal[8], liver cells[9], cervix[10], adenocarcinoma of the esophagus[11], lymphoma[12], among others[13,14].

However, the methods used in the majority of studies involving the separation of high molecular weight proteins by centrifugation of the blood serum ultrafiltration membrane have a high cost.[15] In addition, they require laborious purification of the sample through testing by SPE with resins difficult acquisition, hindering the implementation of the method on a routine analysis.[16]

Thus, a method was developed for protein precipitation of high molecular weight iced organic solvents using acetone, methanol, acetonitrile and isopropanol to recover nine nucleosides, including some modified, which are currently associated with the presence of many diseases, including various malignancies. It was also optimized a method of analysis by LC-MS / MS without the need for extraction of nucleosides by SPE.

II. MATERIALS AND METHODS

Reagents and solutions

All reagents used herein were of analytical purity grade. nine nucleosides standards (Cytidine - C, Thymidine - T, Adenosine - A, Guanosine - G, 2'-DeoxyAdenosine - 2dA, Inosine - I, 5-MethylUridine - 5mU, Xanthosine – X and Uridine - U) and the internal standard 5-fluoridine - IS - were acquired from Sigma-Aldrich (St. Louis, Missouri, EUA) (Fig. 1). All solutions were prepared with deionized water obtained from a Milli-Q system from Millipore (Bedford, MA, U.S.A.). The solutions were filtered in sterile nylon filter Millex 0.20 µm Millipore (Cork, Ireland) before being injected into the equipment.

For the preparation of mobile phase were used formic acid Synth (Diadema, Brazil) and acetonitrile Panreac (Barcelona, Spain). The mobile phases were filtered through

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Millipore membranes (Bedford, USA) PVDF (aqueous phase) and PTFE (organic phase) with 0.22 µm pore. The organic solvents used for protein precipitation were methanol Carlos Erba (Milan, Italy) and acetone and isopropanol Panreac (Barcelona, Spain).

Apparatus

The chromatographic separation was made by using a ultra-efficiency chromatograph (UPLC) Acquity Ultra Performance coupled to a triple quadrupole mass spectrometer Quattro Micro API with electrospray ionization source (ESI) from Waters (Milford, MA, USA).

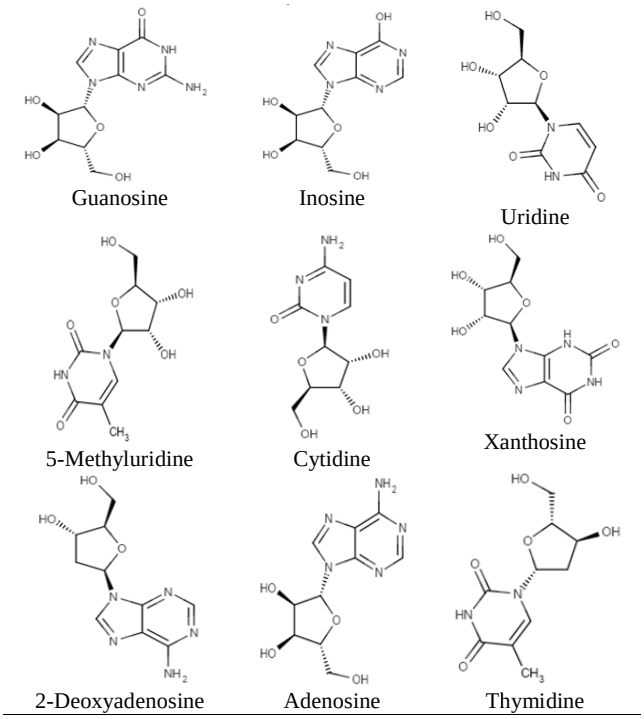

Fig.1The chemical structures of investigated nucleosides.

Sample Preparation

The blood samples of 57 healthy volunteers male and female used in the validation procedure was obtained from the Blood Center, located in the Hospital of Unicamp. The blood was collected in a dry tube and the serum was separated by centrifugation at 4 ° C for 15 minutes at 3000 rpm, and subsequently stored at -80 ° C until analysis.

Liquid chromatography–mass spectrometry (LC–MS/MS)

The chromatographic separation was performed using an ACE C18 HPLC column (Aberdeen, Scotland) with dimensions of 150 x 4.6 mm x 3 micrometers. The flow ratio used was 0.6 $mLmin^{-1}$ and injection volume of 10 µL. The aqueous mobile phase (FA) was composed of 0.10% formic acid and organic mobile phase with 0.10% formic acid in acetonitrile, gradient elution with 95% a 0-4 min 30% 5 min 40% at 6 min and 95% by 8 min, an adaptation of the method the Coulier et al. (2006).[17]

The optimization of the mass spectrometer parameters for nucleosides was made through single injections of analytical standards 0.010 $mmolL^{-1}$. To optimize the precursor ion and confirmation ion, cone voltage and collision energy was used AutoTunyprogram from MassLynx. The ionization source mode was determined as positive mode, capillary voltage 1000 V, source temperature 150 °C and desolvation temperature of 300 °C.

Optimisation of protein precipitation method for the recovery of nucleoside

This 100 µL of the solution of nucleoside standards were added to 0.50 $mmolL^{-1}$ in eppendorf with 400 µL of pool from blood serum of healthy individuals, to obtain a final concentration of 0.10 $mmolL^{-1}$. Thereafter was added 500 µL of cold organic solvent (acetonitrile, methanol, acetone or isopropanol), vortexed for one minute and then centrifuged for 10 minutes at 6000 rpm and 4 ° C. The supernatant was transferred to another Eppendorf being added another 500 µL of the respective cold solvent, and vortexed for one minute and centrifuged in the same conditions. Subsequently, the supernatant was transferred to a 50 mL falcon tube and the contents evaporated in nitrogen flow (30 minutes to acetone and isopropanol; 1 hour for acetonitrile and methanol). The protein precipitation process is described in the flowchart shown in Fig. 2.

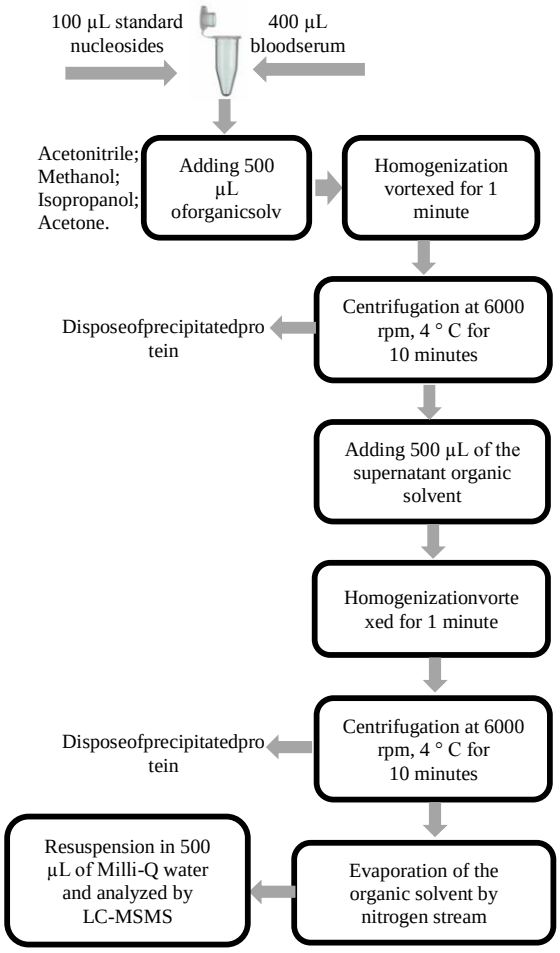

Fig. 2 Flowchart of precipitation of high molecular weight proteins using organic solvent

Ethics

The project was approved by the Research Ethics Committee (CEP) of the Campinas State University, São Paulo - Brazil, with opinion nº. 136.395 / 2012.

Method validation

All validation parameters were evaluated in standard solutions prepared in the biological matrix, i.e. blood serum samples from healthy volunteers. Method validation was carried out in agreement with the parameters specified by the Brazilian National Health Surveillance Agency (ANVISA) for bioanalytical methods: limits of quantification, linearity, precision and accuracy.[18]All assays were performed in triplicate in the biological matrix.

II. RESULTS AND DISCUSSION

Mass method development

In order to obtain better MS conditions, they were optimized with corresponding standard solutions. The positive effect and negative ionization modes on the sensitivity of the assay wasanalitically investigated. The results showed that the ionization of the nucleosides were more efficient in positive mode, consistent with previous reports.[5,12,17,19-23]

For maximum sensitivity after the determination of ionisation mode, the optimization of the conditions of mass spectrometry was carried out through direct injections of individual solutions of analytical standards in the MS. To optimize the precursor ion and fragment ion together with confirmation of the cone voltage and collision energy was used AutoTuny program MassLynx program, carried out automatically by the machine. The capillary voltage was maintained at 1 kV, the source temperature 150 ° C and desolvation temperature to 300 ° C for all analytes. The results of the optimal conditions are shown in Table 1.

TABLE 1 OPTIMIZATION OF THE MASS SPECTROMETER CONDITIONS

	Mean retention time (min)	Ion (m/z)	Fragment (m/z)	Cone (V)	Collision of Energy (V)
5mU	5.11	259	127	20	10
			110*	20	35
I	6.16	269	137	10	10
			110*	10	40
2dA	6.17	252	136	15	10
			119*	15	40
C	2.78	244	112	15	10
			95*	15	40
U	3.64	245	113	15	10
			70*	15	30
X	5.36	285	153	15	10
			133*	15	10
T	6.59	243	127	10	5
			117*	10	10
G	4.43	284	152	15	10
			135*	15	40
A	6.16	268	136	20	15
			119	20	45
SI	4.40	263	131	15	10
			73*	15	25

Chromatography

Optimization of chromatographic analysis aimed at obtaining a quick procedure with a few minutes of running for simultaneous analysis of the nucleosides. The best resolution of the peaks was obtained from an optimization in the gradient method proposed by Coulier et al. (2011)[17], which is used as 0.1% aqueous formic acid and organic phase composed of 0.10% formic acid in acetonitrile (ACN). Thus, initially analyzes were performed with the same gradient proposed by the authors (0.5% B within 2 min, increase to 50% B in 4 minutes, 100% B for 1 minute and finally 100% B for 2 minutes). It was later performed an optimization of the gradient to improve the chromatographic separation of the analytes, and optimized the condition gradient elution with 95% to 30% 0-4 min 5 min 40% at 6 min and 95% by 8 min. Figure 3 shows the chromatogram obtained under the conditions described.

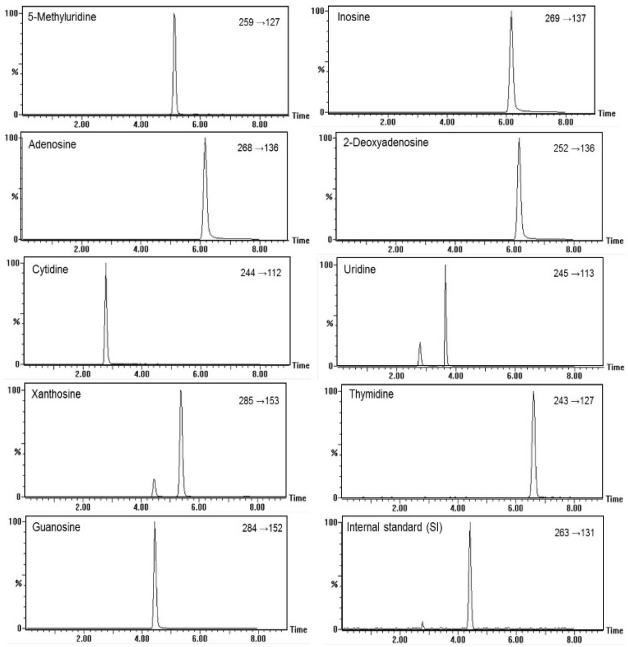

Fig. 3 Representative chromatograms obtained under optimized analysis conditions.

Precipitation of serum proteins with organic solvents

The separation of high molecular weight proteins in blood serum normally are performed by centrifuging with ultrafiltration membrane Millipore 30 kDa for 1 hour at 6000 rpm and 4 ° C. However, the membranes used are costly, difficult to implement the method in routine laboratories, because it should be used by a membrane sample.[24-26]

Thus, precipitation of thehight weight molecular proteins by iced organic solvents was evaluated using acetone, methanol, acetonitrile and isopropanol, as described in the literature as solvents precipitants.[27-29]

The high recovery of analytes are important considerations in the approach of clean-up methods used for purification of samples. However, many conventional strategies for protein precipitation inevitably have contributed to the loss of analytes and / or increasing the laboriousness of the analysis, making it impossible to use in routine analysis.[30]

Among the biological samples purification techniques we can mention the use of iced organic solvents for protein precipitation. Generally, this method is used to purify the proteins present in different biological matrices (serum, plasma or urine) in order to analyze the proteins.[31-33]In this study, the goal was to use the same foundation to eliminate high molecular weight proteins which could interfere with the analysis of the analytes of interest (nucleosides) that have low molecular weight. In this study, different organic

solvents were tested for precipitation of proteins present in blood serum while eliminating interferents for the analysis of nucleosides by HPLC-MS / MS.

In aqueous solution, when the composition of the medium is constant (steady state), the proteins adopt a structure that exposes hydrophilic regions (side chain residue of polar and ionic amino acids) to the aqueous surrounding, allowing the formation of a hydration layer and protecting hydrophobic amino acids therein. However, when the rupture of the hydration layer is carried out by contact of organic solvents, the ions can competitively disrupt ionic interactions on hydrophobic polypeptide demand centers, causing reorganization of water around the protein. Thus, the interior is no longer protected from the solvent, causing various conformational transitions including the weakening of existing interactions, besides favoring hydrophobic interactions, conditioning the aggregation or precipitation of the protein.[30,34]Based on this principle, we tested four organic solvents (methanol, acetone, acetonitrile and isopropanol) to assess recovery of the analytes after the precipitation of the proteins, so that occurred the lowest occlusion loss possible. The results are shown in Table 2.

TABLE 2. RECOVERY OF NUCLEOSIDES AFTER PRECIPITATION OF PROTEINS WITH DIFFERENT ORGANIC SOLVENTS

	Recovery (%) ± standard deviation			
Nucleosídeos	**ACN**	**MeOH**	**ACE**	**ISO**
5mU	81 ±3	76 ±2	79 ±2	43 ±2
I	82 ±3	61 ±1	89 ±2	49 ±3
A	84 ±4	61 ±1	87 ±0,3	48 ±2
2dA	83 ±3	60 ±1	88 ±2	48 ±2
C	2,0 ±0,1	1,9 ±0,1	3,0 ±0,1	2,0 ±0,1
U	57 ±4	68 ±5	73 ±12	48 ±7
X	83 ±2	80 ±1	83 ±2	52 ±1
T	91 ±4	85 ±2	89 ±2	54 ±4
G	73 ±2	65 ±2	79 ±3	48 ±2

ACN-Acetonitrile; MeOH-Methanol; ACE-Acetone; ISO-Isopropanol

Despite the recovery difference of analytes from acetone and acetonitrile have not been high (Table 2), acetone is the most suitable choice, because it takes half the time of evaporation of the ACN associated with evaporation capacity (pressure steam 200 mm Hg at 22.7 °C) exceeding acetonitrile (vapor pressure 100 mm Hg at 27 °C), reducing the time of sample preparation. However, regardless of the solvent used, the recovery of cytidine ranged from 1.9 to 3%. Thus, this method of clean up is not suitable only for separation of cytidine.

Method validation

The calibration curves and lower limit of quantification (LLOQ) of nucleosides are presented in Table 3. The developed method exhibited good linearity with excellent superior a0.9872 for all analytes. The results indicated that the LLOQs were suitable for analytes determination.

TABLE3. CALIBRATION CURVE, CORRELATION COEFFICIENTS AND LINEAR RANGES OF BLANK SERUM SPIKED WITH NUCLEOSÍDEOS.

Compound	Calibration curve	Correlationcoefficient	LLOQ* (µmol/L)	Precision (LLOQ)	Recovery (LLOQ)
U	y=91,746x+1,7801	0,9898	0,005	18,15	52,15
A	y=115,03x-0,0496	0,9960	0,005	11,88	90,49
I	y=118,82x-0,1673	0,9975	0,005	16,77	93,46
G	y=5,5156x-0,0322	0,9888	0,005	14,99	91,15
5mU	y=0,8169x-0,2387	0,9911	0,005	17,56	132,5
X	y=17,781x+0,0928	0,9872	0,005	15,79	113,7
T	y=5,3158x-1,6056	0,9958	1,000	2,073	88,95
2dA	y=118,34x-0,1056	0,9997	0,005	2,010	88,34

*LLOQ, lower limit of quantification.

The accuracy and precision were assessed by samples with five replicates (low, middle and high concentration) on three continuous days. Relative standard deviation (RSD%) was used to evaluate the intra- and inter-day precision and it should not exceed 20%. To assess the accuracy, the relative error (RE%) was calculated according to the formula: RE% = [(assayed value−nominalvalue)/nominal value]x100.

As shown in Table 4, the intra-day and inter-day precisions (RSD) of samples at three different levels were both less than 20% and the accuracies (RE) ranged from − 0,00798% to 11,778%.

TABLE 4. PRECISION AND ACCURACY FOR THE DETERMINING OF NUCLEOSIDES IN SERUM (N = 3 DAYS, FIVE REPLICATES PER DAY).

Compounds	Con. (µmol/L)	Precision (%)		Accuracy (%)
	Spiked	Intra-day	Inter-day	
T	1	9,7904	8,4013	2,5345
	3	9,5519	12,556	7,0966
	5	5,6057	6,3260	-1,3450
5mU	0,275	14,921	12,183	-0,3492
	1,025	14,619	6,2735	-7,5018
	1,525	10,541	14,752	-1,0873
I	0,0050	18,572	19,041	7,5906
	0,025	9,8309	12,578	6,4603
	0,045	4,0136	2,7484	-1,0205
U	0,055	4,4226	10,893	4,4226
	0,525	4,7712	13,494	10,4975
	1,025	4,6516	11,038	-6,3476
2dA	0,0050	17,804	4,9333	2,4557
	0,025	11,2949	23,271	6,9868
	0,045	9,0495	6,3940	-0,5192
A	0,0050	13,663	19,600	2,5052
	0,025	18,663	18,712	4,5130
	0,045	6,2104	3,6670	-2,3976
X	0,0050	12,691	13,200	15,775
	0,025	15,800	10,593	0,00798
	0,045	12,654	6,2309	-0,5109
G	0,525	11,002	11,941	4,2614
	1,025	12,022	5,3875	-11,778
	1,525	2,5518	5,9523	0,54429

Con.: Concentration

III. CONCLUSION

It was developed and validated a method for simultaneous quantification ofnine nucleosides, including some modified in blood serum. This methodology can be used to evaluate the expression of nucleosides as biomarkers for different

diseases including malignancies. Moreover, simultaneous determination of nucleosides in a single run and quick clean up process by protein precipitation by organic solvents makes it compatible with routine analysis in clinical diagnostics laboratories.

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REFERENCES

[1] Seidel A., Brunner S., Seidel, P., Fritz, G.I., Herbarth, O., *BritshJournal of Cancer*, 94, 1726, 2006,

[2] Gehrke, C. W., Kuo, K. C., Waalkes, T. P. & Borek, E. *Cancer Research*, 39, 1150-1153, 1979.

[3] Hall, R.H. *Biochemistry*, 4, 1275-1292, 1965.

[4] Borek, E., Baliga, B.S., Gehrke, C.W., Kuo, K.C., Belman, S., Troll, W., Wallkes, T.P.*Cancer Research*, 37, 3362, 1977.

[5] Cho, S. H.; Choi, M. H.; Lee, W. Y.; Chung, B. C. Clinical *Biochemistry*, 42, 540–543, 2009.

[6] Zheg, Y.F.; Yang, J.; Zhao, X. J.; Feng, B. Kong, H. W.; Chen, Y. J.; Lv, S.; Zheng M. H.; Xu, G. W. *World Journal of Gastroenterology*, 25, 3871-3876, 2005.

[7] Mao, Y., Zhao, X., Wang, S., Cheng, Y., *AnalyticaChimicaActa*,34, 598, 2007.

[8] Feng, B., Zheng, M., Zheng Y., Lu, A., Li, J., Wang, M., Ma, J., Xu, G., Liu, B., Zhu, Z. *Journalof Gastroenterologyand Hepatology*, 20, 1913, 2005.

[9] Jeng, L. B.; Lo, W. Y.; Hsu, W. Y.; Lin, W. D.; Lin, C. T.; Lai, C. C.; Tsai, F. J. *Rapid Communications in Mass Spectrometry*,23, 1543–1549, 2009.

[10] Termine, L.; Villa, L. L.*JornalBrasileiro deDoençasSexualmenteTransmissiveis*, 20, 125-131, 2008.

[11] Djukovic, D.; Baniasadi, H. R.; Kc, R.; Hammoud, Z.; Raftery, D. *Rapid Communications in Mass Spectrometry*,24: 3057-3062, 2010.

[12] Li, H. Y.; Wang, S. M.; Liu, H. M.; Bu, S. S.; Li, J.; Han, D.; Zhang, M. Z.; Wu, G. Y. Journal*Mass Spectrometry*, 44,641-651, 2009.

[13] Markuszewski, M. J., Struck, W., Waszczuk-Jankowska, M., Kaliszan, R., *Electrophoresis*,31, 2010.

[14] Seidel, P.; Seidel, A.; Herbath, O. *Neural Networks*, 20, 646-651, 2007.

[15] http://www.merckmillipore.com/

[16] Buzatto, A. Z., Guedes, S. F., Vidotto, L. T., Oliveira, L. C., Simionato, A. V. C., *Journal Brazilian Chemistry Society*, 24, 904, 2013.

[17] Coulier, L.; Gerritsen, H.; Kampen, J. A. V.; Reedijk, M. L.; Luider, T. M.; Osterhaus, A. D. M. E.; Gruters, R. A.; Brull, L. *Journal of Chromatography B*, 879, 2772-2782, 2011.

[18] Brazilian National Health Surveillance Agency (ANVISA) Resolution RDC 27, from 17/05/2012.

[19] Hsu, W. Y.; Chen, W. T.; Lin, W.; Tsai, F. J.; Tsai, Y.; Lin, C. T.; Lo, W. Y.; Jeng, L. B.; Lai, C. C. *ClinicaChimicaActa*, 402, 31-37, 2009.

[20] Hsu, W. Y.; Lin, W.; Tsai, Y.; Lin, C. T.; Wang, H. C.; Jeng, L. B.; Lee, C. C.; Lin, Y. C.; Lai, C.C.; Tsai, F. J. *ClinicaChimicaActa*, 412, 1861-1866, 2011.

[21] Bond, A.; Dudley, E.; Lemiere, F.; Tutten, R.; Sharkawi, S.; Brenton, A. G.; Esmans, E. L.; Newton, R. P. *Rapid Communications in Mass Spectrometry*, 20, 137-150, 2006.

[22] Yang, J.; Xu, G.; Kong, H.; Zheng, Y.; Pang, T.; Yang, Q. *Journal of Chromatography B*, 780, 27-33,2002.

[23] Yamaoka, N.; Kudo, Y.; Inazawa, K.; Inagawa, S.; Yasuda, M.; Mawatari, K. I.; Nakagomi, K.; Kaneko, K. *Journal of Chromatography B*, 878, 2054-2060, 2010.

[24] D'Avila, F. B.; Pereira, A. G.; Salazar, F. R.; Ferreira, P. L.; Salazar, C. R.; Limberger, R. P.; Froehlich, P. E. *Journal of Pharmaceutical and Biomedical Analysis*, 103, 67-72, 2015.

[25] Petruczynik, A.; Wroblewski, K.; Szultka-Młynska, M.; Buszewski, B.; Karakuła-Juchnowicz, H.; Gajewski, J.; Morylowska-Topolska, J.; Waksmundzka-Hajnos, M. *Journal of Pharmaceutical and Biomedical Analysis*, 127 68-80, 2016.

[26] Jouyban, A.; Sorouraddin, M. H.; Farajzadeh, M. A.; Somi, M. H.; Fazeli-Bakhtiyari, R. *Talanta*, 134,681-689, 2015.

[27] Duellman, S. J.; Burgess, R. R. *Protein Expression and Purification*, 63, 128-133, 2008.

[28] Ingham, K. C. *Methods in Enzymology*, 182, 301-306, 1990.

[29] Burgess, R. R. *Methods in Enzymology*, 208, 3-10, 1991.

[30] Andrew M.J.; Crowell, Mark J.; Wall, Alan A. *AnalyticaChimicaActa*, 796, 48-54, 2013.

[31] Avila, F. B. D'.; Pereira, A. G.; Salazar, F. R.; Ferreira, P. L.; Salazar, C. R.; Limberger, R. P.; Froehlich, P. E. *Journal of Pharmaceutical and Biomedical Analysis*, 103, 67-72, 2015.

[32] Crowe, K. M. *Clinical Biochemistry*, 47, 116-118, 2014.

[33] Jiang, L.; He, L.; Fountoulakis, M. *Journal of Chromatography A*, 1023, 317-320, 2004

[34] Scopes, R.K. 2nd ed., Springer- Verlag, New York, 1987.