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Development and validation of HPLC method to determination of Methotrexate in children oncologic patients

C.C. DE ABREU[1], P.C. PIRES ROSA[2], B. DA COSTA AGUIAR ALVES[1], L. AJAIME AZZALIS[3], F. DE SOUZA GEHRKE[1], E.C. PEREIRA[3], V. BERLANGA CAMPOS JUNQUEIRA[3], F. FERREIRA PERAZZO[3], F.L. AFFONSO FONSECA[1,3]

[1]Laboratório de Análises Clínicas, Faculdade de Medicina do ABC, Santo André, SP, Brasil
[2]Department of Pharmacology, School of Medical Sciences (FCM), University of Campinas (UNICAMP), Campinas, São Paulo, Brazil
[3]Instituto de Ciências Ambientais, Químicas e Farmacêuticas, UNIFESP, Diadema, SP, Brasil

Abstract. – OBJECTIVE: The acute lymphocytic leukemia is a hematopoietic cancer that occurs predominantly in children. Methotrexate is one of the most useful drugs in cancer chemotherapy. The aim of the study was to develop and validate the methodology of high performance liquid chromatography (HPLC) with ultraviolet detection for methotrexate dosage and to determine its concentration in plasma samples from children with leukemia.

PATIENTS AND METHODS: The study included patients from the outpatient care of pediatric oncology at the Faculty of Medicine of ABC carriers in treatment of leukemia. The study was conducted in chromatographic model Agilent 1100 with UV detector at 302 nm and by the method of ELISA microplate reader capable of reading absorbance at 450 nm.

RESULTS: We obtained satisfactory results of selectivity, accuracy, linearity, limit of quantification (LOQ), limit of detection (LOD), precision and robustness and apply the basic criteria for validation as RE No. 899, of May 29, 2003 Guide validation of analytical and bioanalytical National Agency Health Surveillance (ANVISA).

CONCLUSIONS: We conclude that results for linearity/concentration range, precision, robustness, limit of quantification and detection limits are within the acceptance criteria defined by ANVISA and that the developed analytical method is valid and feasible to be used as a tool in monitoring therapy of methotrexate.

Key Words:
Validation, Methotrexate, Children, Oncology.

Introduction

The acute lymphocytic leukemia (ALL) is a hematopoietic cancer that occurs predominantly in children. It is the result of the growth of a type of abnormal non-granular leukocytes, fragile, in blood-forming, tissues especially bone marrow, spleen and lymph nodes. The abnormal cell has little cytoplasm and uniformly round core which resembles of the lymphoblast. The compromised immune response to the hypogammaglobulinemia and to the autoimmunity is not characteristics of acute lymphoblastic leukemia in contrast to other lymphoproliferative diseases, and abnormal cells do not appear to be related with immunologically competent lymphocytes from the blood. The normal bone marrow elements can be moved or replaced in this type of leukemia, but there is no evidence that the basic abnormality involving stem cells differentiate into erythrocytes, granulocytes and megakaryocytes[1].

The term acute lymphocytic leukemia will be used to include the leukemias in children or in adults that sometimes are referred to as acute lymphoblastic leukemia, stem cell leukemia acute leukemia acute indifference. The clinical features of ALL are similar to those described for acute leukemia in general and are mostly related to fever, often by infection, lymphadenopathy, splenomegaly, anemia, and bleeding from thrombocytopenia[1].

Methotrexate (MTX-N-[4-{[(2,4-diamino-6-pteridinyl) methyl] methylamino} benzoyl]) is one of the most useful drugs in cancer chemotherapy; it is a weak bicarboxylic acid with pKa 4.8 and 5.5 structurally related to folic acid (pteroylglutamic) and to the aminopterin. MTX belongs to the class of inhibitors of high selectivity, with primary target dihydrofolate to tetrahydrofolate, acting as competitive with dihydrofolate by binding in the active sites of the en-

Corresponding Author: Beatriz da Costa Aguiar Alves Reis, MD; e-mail: bcaalves@uol.com.br

zyme. The tetrahydrofolate is the precursor of the active form of folate cofactor, necessary for synthesis of thymidylate, purines, methionine and serine. It is assumed that cells exposed to MTX die due to the absence of reduced folates affecting the synthesis of DNA, RNA and proteins, like all the metabolic reactions dependent on the methyl group[2]. However, beside the benefits in cancer treatment, MTX presents some side effects, such as bone marrow suppression, alopecia, stomatitis and development of hepatic fibrosis or cirrhosis[3], and its use should be monitored.

The aim of the study was to develop and validate a high performance liquid chromatography (HPLC) methodology with ultraviolet detection for drug dosage and determine the concentration of methotrexate in serum samples from children with leukemia who make therapeutic use of this drug and comparing the data from the ELISA method.

Patients and Methods

Biological Samples

The study included 10 patients in the outpatient care of pediatric oncology at the Faculty of Medicine of ABC carriers in treating leukemias, maintenance and consolidation from 1.0 g/m^2 after 24 and 48 hours of infusion were harvested 5.0 mL whole blood, between 0-18 years of chemotherapy treatment with ≥ 25 mg/m^2 dose of methotrexate. Exclusion criteria were coexistence of rheumatic disease and others co-morbidity serologicals. For determination and validation of methotrexate by HPLC method the collect was made by venipuncture in tube without additive (dry). After collect, the samples were processed at the Clinical Laboratory of the Faculty of Medicine of ABC and were centrifuged at 2500 g for 10 minutes. After centrifugation, the serum was separated with the assistance of a Pasteur pipette and was stored at –80°C until the time of determination by HPLC.

Chromatographic Conditions

The chromatographic study was conducted in an Agilent 1100 model UV detector at 302 nm and the column (stationary phase) Zorbax Eclipse XDB-C8 150 x 4.6 mm, particle size of 3.5 mm at a temperature of 30°C, mobile phases solvent, and diluent pH buffer 6.0: acetonitrile in the ratio of 93:7, flow: 1.0 mL/min; volume of injection: 20 µL and run time of 30 minutes. For the preparation of the mobile phase and solvent, 7.8 g of citric acid and 17.9 g of anhydrous dibasic sodium phosphate ($Na_2HPO_4.12H_2O$) were used and transferred into a 1000 mL volumetric flask. It was added 800 mL of ultrapure water, and after the solubilization of the salts, it was completed the flask's volume with ultrapure water. Mixed approximately 930 mL of this buffer and 70 ml of acetonitrile, filtered through a membrane HV 0.45 µm.

Reagents

We used the following reagents and standards: Citric Acid Tedia® (Rio de Janeiro, Brazil) grade 99.5% lot: VB0947R1, Cap Dibasic Tedia® (Rio de Janeiro, Brazil) grade 99.0%, Lot: ZK0169, reference standard Libbs lot: PS83010/12 content: 87.6%, Control Lab Biochemistry I lot: TGH-3P in vitro use – potentially infecting ANVISA/Reblas No. PROF001 and Randox – Drug Control Level 2 (TDM Control 2), Membrane Millipore® HV 0.45 µm (Millipore, Bedford, MA, USA).

Preparation of Standard Solution of Methotrexate (Libbs)

Ten mg of Libbs (Sao Paulo, Brazil) methotrexate from the reference standard were diluted in 100.0 mL of diluent and allowed in ultrasound until complete dissolution, stirring occasionally; the volume was completed with the mobile phase until it reached the final concentration of 0.5 µg/ mL.

Standard solution Lab Biochemistry I and Randox (Drug control level 2) was resuspended by adding 5.0 ml of diluent and allowed in ultrasound until complete dissolution.

Quantitative Determination of the Concentration of Methotrexate in Plasma by ELISA

The methotrexate was determined by competitive enzyme immunoassay. Wells were pre-incubated with murine monoclonal antibody against methotrexate. Standard Methotrexate, Buffer containing Blue Dye (diluent RD1-78), Calibrator (RD5P diluent), Wash Buffer, Substrates: Hydrogen Peroxide (color reagent A) and Chromogen-tetramethylbenzidine (color reagent B) and Stop solution (sulfuric acid) were used.

The plasma was collected using heparin as an anticoagulant and centrifuged for 15 minutes (approximately 1000 g) protected from light. Initially, 100 mL of diluent were added to each well, followed by addition of 50 mL of the respective

sample wells. After incubation, the volume was aspirated from the wells and they were subjected to washing with 400 mL of wash buffer (automatic washer). At the end of the last rinse added 200 mL of Conjugate to each well, the plate was incubated with the help of stirrer. After successive washings, was added 200 µL of substrates solution into each well and the plate was incubated again for 30 minutes protected from light (local temperature). Finishing the period of incubation, 50 uL of stop solution were added to each well. The coloring which can vary from blue to yellow was observed, and the optical density of each well was determined using a microplate reader set to 450 nm.

Validation of Method

The items validation followed RE No. 899, of May 29, 2003 of the Guide to validation of analytical and bioanalytical methods from National Agency for Sanitary Vigilance ANVISA[4].

Selectivity

This test is resulted from the comparison of the chromatograms with the following solutions: solvent/mobile phase, pattern of methotrexate, patient samples and standard samples Biochemistry Lab I and Randox – Drug Control Level 2 (TDM Control 2).

Linearity, Quantification Limit and Detection Limit

The percentage limits for the quantification of the amount of analyte were contained in the range of 50% to 120% of theoretical concentration of the test. We used 5 concentrations levels to obtain the analytical curve: 0.25 µg/mL; 0.40 µg/mL; 0.50 µg/mL; 0.55 µg/mL; 0.60 µg/mL (representing 50%, 80%, 100%, 110% and 120% of theoretical concentration). To determine the theoretical values of limit of quantification (LOQ) and limit of detection (LOD) should be used the values of standard deviation of the responses and the inclination taken from statistical curve of linearity increased by two points with concentrations below 50% of the target value. These two points additional chosen are 5% and 10%, corresponding to 0.03 µg/mL e 0.05 µg/mL.

For methotrexate stock solution was transferred accurately weighed 10.0 mg of methotrexate reference standard for Libbs 100.0 mL volumetric flask. Added 60.0 mL of diluent and allowed in ultrasound until complete dissolution, stirring occasionally. It was completed the volume with diluent. Pipetted 5.0 mL of this solution to 100.0 mL volumetric flask (final concentration: 0.005 µg/mL).

Based on the standard error and the slope the limit of detection (LOD) is expressed as Equation 1.

$$LOD = \frac{3 \cdot 3\sigma}{S}$$

Where:

σ – standard error (Note: The standard error is estimated by statistical regression curve of linearity);

S – slope of the calibration curve of the analyte.

Based on the standard error and the slope, the limit of quantification (LOQ) is expressed as Equation 2:

$$LOQ = \frac{10\sigma}{S}$$

Where:

σ – standard error (Note: The standard error is estimated by statistical regression curve of linearity);

S – slope of the calibration curve of the analyte.

Accuracy

The values of the concentration of methotrexate used in the study to content were: 0.40 µg/mL; 0.50 µg/mL e 0.60 µg/mL (80%, 100% and 120% of theoretical content). Were made 9 (nine) determinations covering the linear range of the procedure: 3 (three) concentrations, with three (3) replicates each. For the methotrexate stock solution was transferred accurately weighed 10.0 mg of methotrexate reference standard for Libbs 100.0 mL volumetric flask. Added 60.0 mL of diluent and allowed in ultrasound until complete dissolution, stirring occasionally. It was completed to volume with diluent. Pipetted 5.0 ml of this solution to 100.0 mL volumetric flask (final concentration 0.005 µg/mL).

Precisions

We used the concentration levels of 0.50 mg/mL (corresponding to 100% of theoretical concentration) where consecutive injections were performed.

Robustness

The robustness study was conducted modifying the analytical method with the following conditions: time, temperature, of the column, mobile phase proportions and flow system.

Results

Selectivity

After injections of the diluent, standard, patient sample, standard Randox – Drug Control Level 2 and standard Biochemistry Lab, we noticed that no peak (impurities) interfered with the retention time of the active methotrexate, thus ensuring purity and quantification of active methotrexate. Figures 1 to 5 show specificities reached from each sample studied.

Linearity, Limit of Quantification and Detection Limit

After realization of the linearity curve, a regression of $R \geq 0.98$ was obtained as shown in Table I, thus, showing a linear method.

Accuracy

The recoverable amount of each individual concentration in this study did not exceed more than 15% (shown in Table II) as the criterion RE No. 899, of May 29, 2003 Guide for validation of analytical methods and bioanalytical National Agency for Sanitary Vigilance (ANVISA)[4].

Precision

For accuracy of the system the same standard solution was injected six times in a row, the method accurately injected the six preparations and intermediate precision was evaluated by the results of standard solutions, obtained by two analysts. The relative standard deviation was ≤ 15.0% for each precision (Table III).

Robustness

For the robustness analysis of chromatographic, conditions such as temperature, composition and flow of the mobile phase were changed. There was also prepared with standard Libbs 100% and injected with different times, so testing the stability of the sample (Table IV). For the temperature condition it was observed that there was poor recovery of the asset to the condition of mobile phase composition was observed that the active methotrexate does not match very well with this change, completely modifying the retention time of the asset and thus failing quantifies it, since for the condition of changing flow of the mobile phase to asset recovery varied a little more recovery when dealing to the reduction of the flow and slightly less of recovery when dealing to the increasing of the flow.

Dosages

Similar concentrations were used when compared to data from pediatric patients, undergoing infusion protocol of 4 g/m^2 for 24 hours, with the highest concentrations ranging between 27 and 150 μM for patients between 0-6 months of age and to patients of 7-12 months of age concentrations of 26-140 μM[5,6] (Table V). Data also indicate that younger patients have a higher elimination capacity than older patients, and systemic clearance of methotrexate decreases due to age, therefore higher plasma concentrations in patients with advanced age[5].

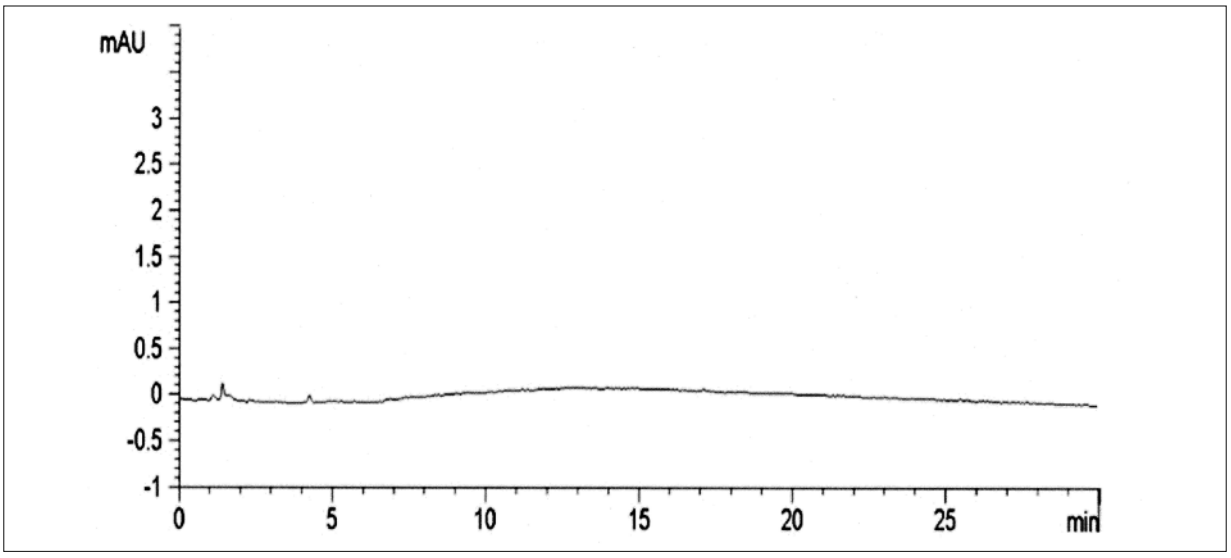

Figure 1. Specificity – Phase chromatogram mobile phase/diluent.

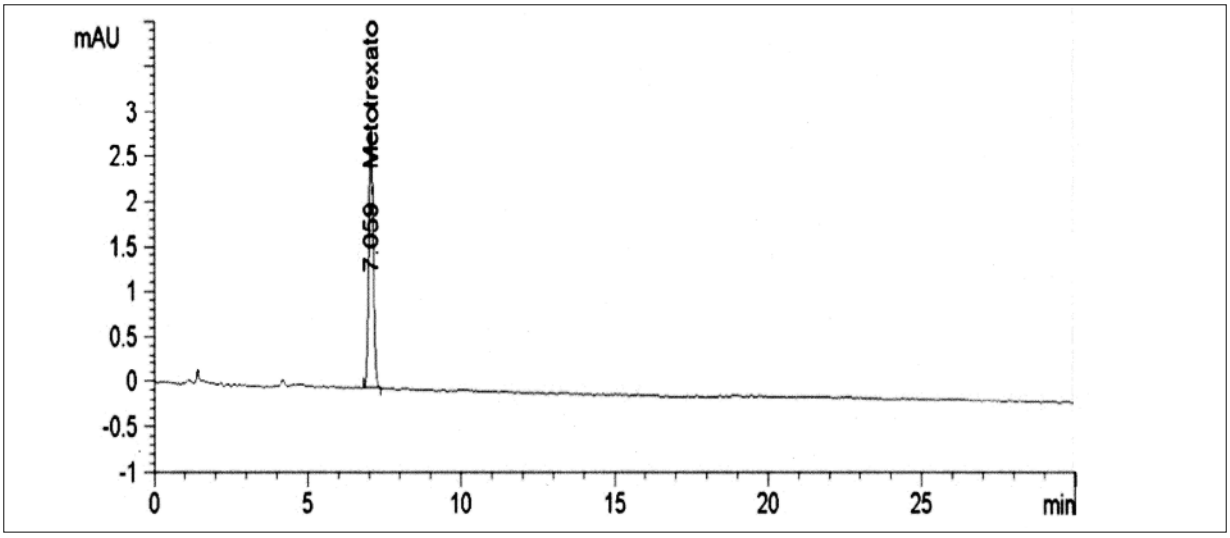

Figure 2. Specificity – Standard chromatogram Libbs.

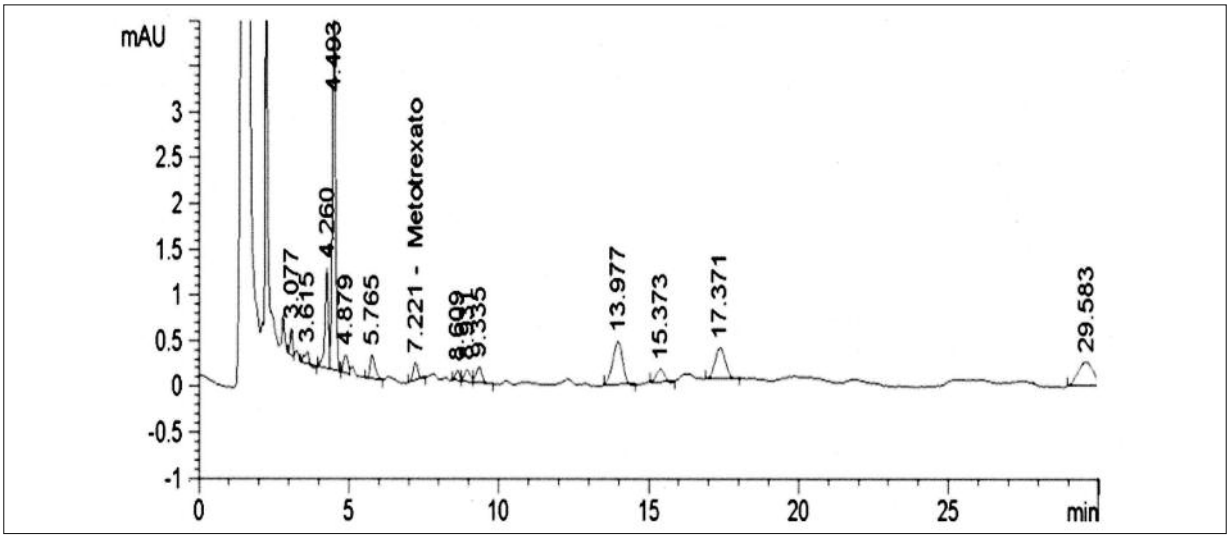

Figure 3. Specificity – Sample chromatogram (Patient).

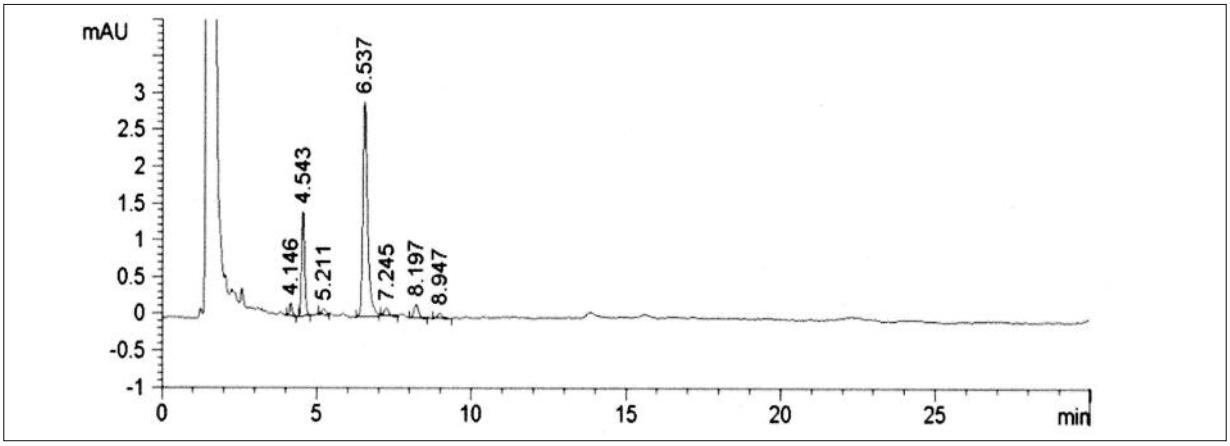

Figure 4. Specificity – Standard chromatogram Randox – Drug Control Level 2.

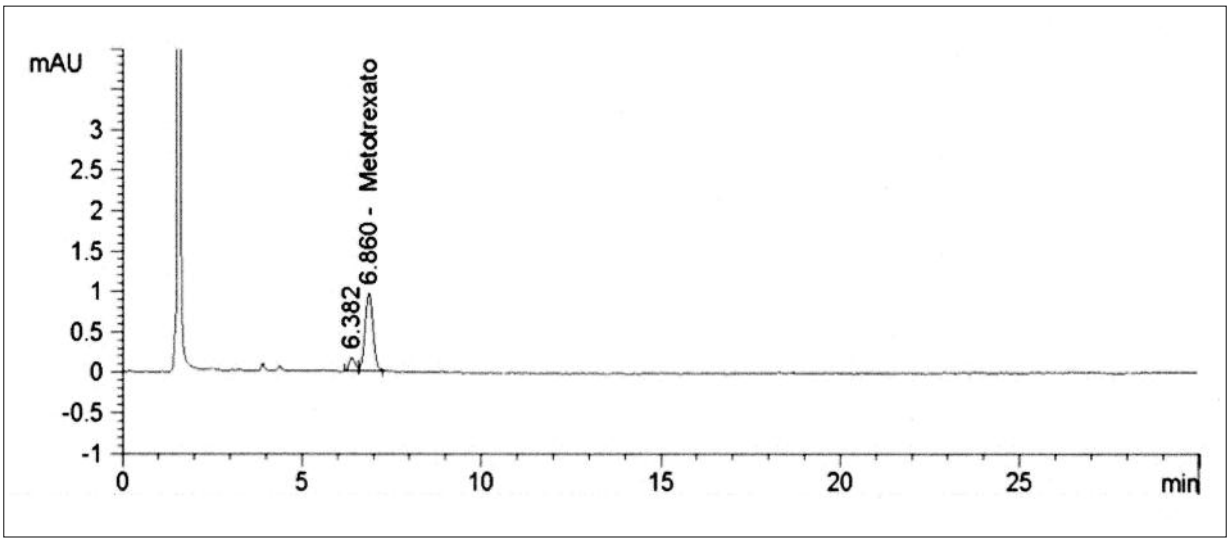

Figure 5. Specificity – Cromatogram Lab Bioquimica I.

Table I. Linearity, LOD and LOQ of MTX.

Compound	Linearity range µg/mL	Correlation coefficient	Area/concentration average ratio-RSD	LOD (µg/mL)	LOQ (µg/mL)
Methotrexate	0.0263-0.6312	0.9999	32.08 4.11%	0.026	0.0090

Table II. Data accuracy of MTX.

Level (%)	Concentration added (µg/mL)	Concentration found (µg/mL)	Recovery (%)	RSD (n=3)	Average Recovery (%)
80	0.4204	0.4011	95.41	2.16	96.29
	0.4072	0.4038	99.17		
	0.4264	0.4021	94.30		
100	0.5255	0.5110	97.24	1.73	97.35
	0.5090	0.5063	99.47		
	0.5330	0.5082	95.35		
120	0.6306	0.6001	95.16	2.35	97.33
	0.6108	0.6138	100.49		
	0.6396	0.6161	96.33		

Table III. Precision data system, method and intermediate of MTX.

Precision	Compound	Concentration (µg/mL)	Retention time (min)	RSD (%)	Tailing factor	Plates number	K'	Assay (%)
Method*	Methotrexate	0.526	6.9	0.1	1.1	4556	2.6	99.2%
System**		0.521	6.9	0.2	1.1	4551	2.7	99.9%
Intermediate**		0.524	6.9	0.2	1.1	4796	2.6	99.8%

*Average of 6 injections, **Average of 12 injections; Value of F calculated: 0.91; F tabulated: 2.82 to 95% probability.

Table IV. Data obtained from the realization of the robustness.

Parameter	Variation	Recovery (%)	RSD (%)
Temperature (ºC)	28	98.6	0.00
	32	98.6	
Mobile Phase (Buffer: ACN)	88:12	Not observed peak	
	98:2		
Flow rate (mL/min)	1.05	94.1	4.90
	0.95	103.9	
Stability (Hours)	0	99.8	0.20
	2	99.6	
	6	100.1	
	12	99.4	
	24	99.7	

Discussion

The acute lymphocytic leukemia is a hematopoietic cancer that occurs predominantly in children, and Methotrexate (MTX) is one of the most useful drugs in chemotherapy; the anti-proliferative effects of MTX explain many side effects of MTX, such as bone marrow suppression, alopecia, stomatitis and development of hepatic fibrosis or cirrhosis[3]. So, the aim of the study was to develop and validate a high performance liquid chromatography (HPLC) methodology with ultraviolet detection for drug dosage and determine the concentration of methotrexate in plasma samples from children with leukemia. For this, several consecutive tests were required, changing the chromatographic conditions such as length, thickness of the column (stationary phase), flow and composition of the mobile phase. After numerous tests, we obtained satisfactory results that apply the basic criteria for validation as RE No. 899, of May 29, 2003 Guide for validation of analytical and bioanalytical National Agency for Sanitary Vigilance (ANVISA).

Table V. Determination of MTX by HPLC and ELISA methods in children samples.

Samples	Method	
	ELISA (µg/mL)	HPLC (µg/mL)
1	< 0.1	0.08
2	Not dosed	0.05
3	Not detected	0.05
4	< 0.1	0.12
5	Not dosed	0.14
6	Not detected	0.06
7	Not dosed	0.07
8	< 0.1	0.06
9	0.13	0.09

Concerning retention times of MTX, in published studies it vary according to applied technique, extraction, concentration and other factors, but with the technique used in this study, the RT MTX has the same times[7]. The recoverable amount of each individual concentration in this study did not exceed more than 15%. Previously published works showed the variation coefficients of about 15%, while the lowest values were 2%[5]. Aboleneen et al[8] during the process of validation of analytical technique, found a variation coefficient of 14.4% for the control of low concentration. Chládek et al[9] had demonstrated that methotrexate was stable under freezing conditions at –80°C to –20°C, however, this study demonstrated that methotrexate behaved very well during a period of stability for 24 hours, showing very good recoveries in their times of stability.

By presenting the faster elimination rate in pediatric patients, this study proved that the method is efficient as well as safe and effective for detecting low concentrations of methotrexate in human plasma after 24 hours. This indicates the risk of late toxicity and enables the monitoring and evaluation of physicians in patients with ALL.

The developed and validated analytical method for the analysis of methotrexate in human plasma proved to be very effective both for the detection and quantification of the drug; the use of HPLC technique allowed the detection of low dosage in pediatric patients enabling further evaluation in relation to ELISA.

In relation to the specificity, no peak had the same range of methotrexate TR interfering it with good resolution of the peaks adjacent to methotrexate. Linearity in the results of the linearity curve of methotrexate was $R \geq 0.98$. The accuracies obtained for the method, the system

and the intermediate accuracies resulted with a DPR of less than 15.0% and the robustness results obtained show that the sample is unstable varying proportionally under the conditions applied during the test.

With this, the results for linearity/concentration range, precision, robustness, limit of quantification and detection limit are within the acceptance criteria defined by Resolution RE No. 899 of May 29, 2003 Guide of validation analytical methods and bioanalytical from National Agency for Sanitary Vigilance[4].

Conclusions

The development of the analytical method of methotrexate therapy in therapeutic patients with acute lymphocytic leukemia is valid and feasible to be used as a tool in monitoring therapy of methotrexate.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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