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Different Analytical Method used for the Estimation of Methotrexate in Rheumatoid Arthritis

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ABSTRACT

Rheumatoid Arthritis (RA) is an inflammatory autoimmune disease characterized by inflammatory polyarthritis, which affects peripheral joints, especially the small joints of the hands and feet. Rheumatoid arthritis is an autoimmune disorder which occurs when our immune system mistakenly attacks own body's tissues. Chronic untreated inflammation may promote joint disintegration and joint destruction. It was more often reported in the females as compared to the males and may be caused at any stage of life. The various drug are used in the treatment of Rheumatoid arthritis included the non-steroidal anti-inflammatory drugs, synthetic and biological Disease-Modifying Antirheumatic Drugs (DMARDs), analgesic agents and non-pharmacological measure seeking to alleviate pain, reduce the damage and preserve the joint function but Methotrexate is the most effective drug used in the treatment of rheumatoid arthritis. It must be given in single or in combination. It will help ease symptoms like joint pain, fatigue, redness, and swelling. A large number of methodologies including high performance liquid chromatography, UV-visible, liquid chromatography-mass spectroscopy, mass Spectrophotometry, capillary electrophoresis and electrochemical methods are used for the determination of methotrexate. The various analytical methods were applied directly and easily for the analysis of the Pharmaceutical formulations. The methods were completely validated as per the ICH Q1A (R^2) guidelines. Thus this strategy can be safely utilized for the standard quality control analysis of Methotrexate.

Keywords: Methotrexate; Rheumatoid arthritis; High performance liquid chromatography; UV Spectroscopy

INTRODUCTION

Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is an inflammatory autoimmune disease characterized by inflammatory polyarthritis, which affects peripheral joints, especially the small joints of the hands and feet. Rheumatoid arthritis is an autoimmune disorder which occurs when our immune system mistakenly attacks own body's tissues. Chronic untreated inflammation may promote joint disintegration and joint destruction [1]. It was more often reported in the females as compared to the males and may be caused at any stage of life. Rheumatoid arthritis is a chronic inflammatory disorder that can affect more in joints. In certain peoples, the condition can harm a wide variety of body frameworks, including the skin, eyes, lungs, heart and blood vessels [2].

LITERATURE REVIEW

In contrast to the mileage harm of osteoarthritis, rheumatoid joint inflammation influences the covering of your joint, causing a painful swelling that give result in bone disintegration and joint deformity [3].

Signs and Symptoms of Rheumatoid Arthritis May Include

- Fatigue, fever and loss of appetite.

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• Early rheumatoid joint pain can affect your little joints first-especially the joints that attach your fingers to your hands and your toes to your feet.

• Warm, swollen joints.

• As the sickness advances, symptoms mainly spread to the wrists, knees, ankles, elbows, hips and shoulders. In many cases, side effect occurs in same joints on both sides of your body **(Figure 1 and Table 1)**.

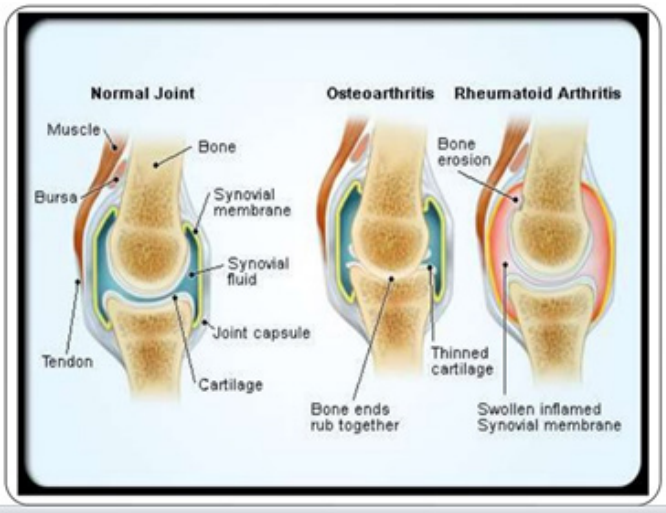

Figure 1: The inflammation related with rheumatoid arthritis can damage other parts of the body as well.

Table 1: DMARDs in RA are those compounds for which an inhibiting effect on radiographic progression has been demonstrated.

Drugs used in the treatment of RA
Conventional synthetic DMARDs
Hydroxychloroquine
Leflunomide
Methotrexate
Sulfaslazine
Targeted synthetic DMARDs
Baricitinib
Tofacitinib
Biological DMARDs
TNF alpha inhibitors
Adalimumab, Golimumab, Infliximab
Certolizumab pegol
Etanercept
Anti-B-cell (CD20)
Rituximab
Anti-T-cell costimulation (CD80, CD 86)
Abatacept
Anti-IL-6
Sarilumab
Tocilizumab

Methotrexate

Methotrexate (MTX) is currently the most frequently used drug in the treatment of Rheumatoid Arthritis (RA). Various combinations of DMARDs have been tried, most generally methotrexate as the anchor drug [4]. Regarding the route of administration of MTX there is some proof that the parenteral route, frequently performed subcutaneously, has some extra advantages over the oral route. In MTX monotherapy, dosages up to 30 mg/week are currently utilized [5].

Main Combination of Methotrexate

There are three main combinations that play an important role in rheumatoid arthritis, Methotrexate+Sulfaslazine (SSZ)+Hydroxychloroquine, Methotrexate+Leflunomide (LEF), Methotrexate+Leflunomide Methotrexate, Methotrexate+biologics (abatacept, adalimumab, etanercept, infliximab, rituximab, tocilizumab)+tofacitinib and other new compounds which block the Interleukin 6 (IL6) receptor or T-cell activation and delete B cells. Regarding clinical efficacy, methotrexate monotherapy has performed correspondingly well in correlation with biologic mono-therapy, both normally joined with glucocorticoids [6]. In any case, basic harm is typically inhibited to an essentially more prominent degree with the biologics. The combination of MTX with biologics has demonstrated better than either operator alone in all perspectives. Current strategic regimens which focus on precise approaches to bring patients into reduction all include MTX as first choice **(Figure 2)** [7].

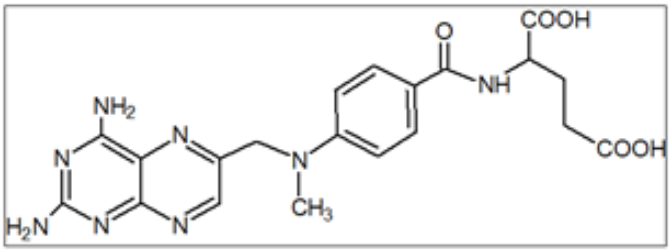

Figure 2: Methotrexate.

Chemical Structure

• **Molecular formula:** $C_{20}H_{22}N_8O_5$

• **Molar mass:** 454 g/mol

• **Melting point:** 193°C-195°C

• **IUPAC Name:** (2S)-2-[[4-[(2,4-diaminopteridin-6-yl) methyl-methylamino] benzoyl] amino] pentanedioic acid.

• **Solubility:** Soluble in dilute solution of alkali hydroxides and carbonates and slightly soluble in dilute hydrochloric acid.

• **Color/Form:** Orange-brown, crystalline powder.

• **Uses:** Methotrexate is used to treat severe psoriasis and rheumatoid arthritis.

• It may also be used to control juvenile rheumatoid arthritis.

• Mechanism of action **(Figure 3)**.

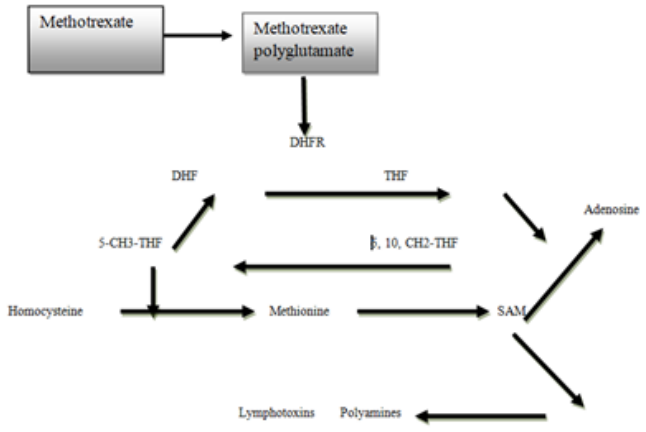

Figure 3: Mechanism of methotrexate.

• **Absorption:** Methotrexate has a bioavailability of 64%-90%; however this reduction at oral doses above 25 mg due to saturation of the carrier mediated transport of methotrexate. Methotrexate has a Tmax of 1 hrs to 2 hrs. oral doses of 10 µg-15 µg arrive at serum levels of 0.01 µM-0.1 µM.

• **Volume of distribution:** The volume of distribution of methotrexate at consistent state is roughly 1 L/kg.

• **Protein binding:** Methotrexate is 46.5%-54% bound to plasma protein.

• **Metabolism:** Methotrexate is metabolized in the liver as well as in tissues.

• **Adverse effect:** Headache, dizziness, anxiety, nausea, vomiting and insomnia.

• **Half-life:** The half-life of low dose methotrexate is 3 hrs to 10 hrs in adults. The half-life for maximum dose of methotrexate is 8 hrs to 15 hrs.

UV Method

The estimation of methotrexate was developed a simple, precise, accurate, cost effective stability indicating by UV Spectrophotometry method shows highest λ max at 303 nm. Beer's law (linearity response) was found over a concentration range of 2 µg/ml-10 µg/ml with good correlation coefficient (r^2 =0.9987) and the estimation of standard deviation were good low and the recovery studies were near to 100 [8]. The Spectrophotometry method was approved as per the ICH Q1A (R^2) guidelines. Thus this strategy can be safely utilized for the standard quality control analysis of Methotrexate [9].

A simple, delicate, specific fast Spectrophotometry strategy was produced for the assurance of post synaptic α1-Adriano receptor antagonist for the estimation of Methotrexate in pure form and pharmaceutical formulations depends on the oxidative coupling response with MBTH reagent, at PH-4.0 which is extractable at 610 nm. Beer's law is obeyed in the concentration ranges 5 µg/ml-30 µg/ml. This developed method was applied directly and easily for the analysis of the Pharmaceutical formulations [10]. In this method R.S.D was found to be 0.2604% and Recovery 98.65% respectively. The method was completely validated as per the ICH Q1A (R^2) guidelines. The obstructions of different ingredients and excipients were not watched. The repeatability and the presentation of the demonstrated strategy were built up by point and internal hypothesis and through recovery studies [11].

Two basic, exact and economical UV strategies have been produced for estimation of Methotrexate in bulk formulation [12].

• **Method A:** Includes estimation of UV absorbance in zero order derivatives at 259 nm.

• **Method B:** Deals with Area Under Curve measurement (AUC method), which involves the calculation of integrated value of absorbance with respect to wavelength between 256 nm-262 nm.

The drug follows Beer-Lambert's law in the concentration range of 3 µg/ml-10 µg/ml in both the methods. Results of analysis were validated statistically and were found to be satisfactory. Thus proposed methods can be successfully applied for estimation of Methotrexate [13].

HPLC Method

A straight, economic, specific, exact, and precise high performance chromatography strategy for the investigation of methotrexate in bulk drug formulations was created and validated in the present study. The mobile phase consists of a mixture of acetonitrile and potassium dihydrogen orthophosphate in the proportion of 92:8, at the pH of 6.0 ± 0.05 with sodium hydroxide solution. It was found to give a sharp peak of methotrexate at a retention time of 4.517 min. HPLC analyzing of methotrexate was done at a wavelength of 303 nm with a flow rate of 1.4 ml/min. The linear regression information for the calibration curve showed a good linear relationship with a regression coefficient of 0.999 in the concentration range of 50 µg/ml to 150 µg/ml. This created strategy was utilized with a high degree of precision and accuracy for the examination of methotrexate [14]. The created technique was approved for accuracy, precision, robustness, detection and quantification limits in accordance with as per according to the ICH rules. The wide linearity range, accuracy, sensitivity, short retention time and composition of the mobile phase indicated that this technique is better for the measurement of methotrexate [15].

A straight, economic, specific, exact, and precise high performance chromatography strategy for the investigation of methotrexate in bulk drug formulations. The estimation was carried out on Enable C18 (250 mm × 4.6 mm, 5 µm) column by using double distilled water; acetonitrile was present in the ratio of 80:20 (v/v) at pH 3 with Formic acid as mobile phase. The flow rate was found to be 1.0 ml/min and the effluent was detected by UV detector at 211 nm. The retention time was found to be 3.28 min and linearity was observed in the concentration range of 8 µg/ml-60 µg/ml. The percentage recovery was in good agreement with the labelled amount in the pharmaceutical formulations and the created technique was approved for accuracy, precision, robustness, detection and quantification limits in accordance with as per according to the ICH rules [16].

A straight, economic, specific, exact and precise high performance chromatography strategy for the investigation of Methotrexate (MTX) Entrapment Efficiency (EE) in polymeric nanocapsules using reversed-phase high-performance liquid chromatography. The technique used a RP-C18 Shimadzu Shimpack CLC-ODS (150 mm × 4.6 mm, 5 µm) column with mobile phase of water-acetonitrile-tetrahydrofuran (65:30:5 v/v/v; pH 3.0) at a discharge rate of 0.8 ml/min. The eluate is checked with a UV detector at wavelength 313 nm. The parameters used in the validation method are linearity, specificity, precision, accuracy and Limit of Quantitation (LOQ). The linearity is assessed by a calibration curve in the concentration range of 10 µg/ml-50 µg/ml and exhibited a correlation coefficient of 0.9998. The polymers (PLA or PLA-PEG), oil and surfactants utilized in the nanocapsule formulation did not interfere with analysis and the recovery was quantitative [17]. The intra and inter-day assay relative standard deviation were under 0.72%. Results are good and the method proved to be a satisfactory for the assurance of methotrexate in nanocapsules formulations [18].

LC-MS/MS Method

Individualized treatment is an ongoing methodology expecting to indicate measurements routine for every patient as per its

hereditary state. Methotrexate (MTX) is a folic acid analogue. Its cytotoxic impact comprises principally in hindering of the catalyst dihydrofolate reductase. MTX is utilized for treatment of intense lymphoblastic leukemia, psoriasis, rheumatoid joint inflammation and so on. Therapeutic monitoring of MTX is performed due to prediction of its impact and toxicity which are relied upon the sum in serum [19]. A critical threshold level predicting irreversible harm of healthy cells is 0.05 µmol/L. Dose regimen of antidote leucovorin (folinic acid) is balanced dependent on the serum level of MTX. The created strategy was approved for the evaluation of methotrexate and its metabolite 7-hydroxymethotrexate by LC-MS/MS with time of examination 4 minute. The concentrations of medication were determined in human serum. Sample preparation includes the precipitation of protein utilizing trichloroacetic acid. We used two calibration curves for quantification because the therapeutic range of MTX is wide. Low calibration curve was applied for concentrations range between 0.05 µmol/L-1 µmol/L and high calibration curve for concentrations from 1 µmol/L to 150 µmol/L. Then we tested repeatability, reproducibility and accuracy on three concentration levels. The coefficient of variation was less than 15% and the recovery was in the range from 95.7% to 113.2% for MTX and its main metabolite [20].

Methotrexate (MTX) is a folic acid analogue. Its cytotoxic effect consists primarily in blocking of the enzyme dihydrofolate reductase. MTX is indicated for treatment of acute lymphoblastic leukemia, psoriasis, rheumatoid arthritis etc. Cancer chemotherapy requires continuous monitoring of the plasma concentration levels of active forms of cytotoxic drugs and subsequent dose adjustment. In order to attain optimum therapeutic efficacy, connection to pharmacogenetics information is significant [21]. In this method, a specific, accurate and sensitive Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) was developed for estimation of Methotrexate (MTX), 6-Mercaptopurine (MP) and its metabolite 6-Thioguanine nucleotide (TG) in human plasma. Based on the fundamental character of the considered compounds, solid phase extraction using a strong cation exchanger was found the ideal way to achieve good extraction recovery. Chromatographic separation was done utilizing RP-HPLC and isocratic elution by acetonitrile 0.1% aqueous formic acid (85:15 v/v) with a flow rate of 0.8 ml/min at 40°C. The detection was performed by tandem mass spectrometry in MRM mode by mean of electrospray ionization source in positive ionization mode. Analysis was done within 1.0 min over a concentration range of 6.25 ng/ml-200.00 ng/ml for the studied analytes [22]. Validation was done by FDA guidelines for bioanalytical method validation and satisfactory results were obtained. The applicability of the test for the observing of the MTX, MP and TG and resulting application to personalized treatment was exhibited in a clinical study on children with Acute Lymphoblastic Leukemia (ALL). The results confirmed the requirement for implementation of reliable analysis tools for therapeutic dose adjustment [23].

Juvenile Idiopathic Arthritis (JIA) and Juvenile Dermatomyositis (JDM) are chronic inflammatory disorders that influence children; they have potentially serious consequences for example joint destruction and disability. The most huge, first line treatment for both these diseases in children and young people is methotrexate. Dried Blood Samples (DBS) [spiked or patient samples] were set up by applying blood to Guthrie cards which was then dried at room temperature [24]. This method used 6 mm disks punched from the DBS samples (equivalent to approximately 12 µl of whole blood). The simple treatment procedure depends on protein precipitation utilizing perchloric acid followed by solid phase extraction using MAX cartridges. The isolated sample was chromatographed using a reverse phase system involving an Atlantis T3-C18 section (3 µm, 2.1 mm × 150 mm) preceded by Atlantis monitor segment of coordinating science. Analytes were exposed to LCMS analysis using positive electrospray ionization. The method was linear over the range 5 nmol/L-400 nmol/L. The limits of detection and quantification were found to be 1.6 nmol/L and 5 nmol/L for individual polyglutamate and 1.5 nmol/L and 4.5 nmol/L for total polyglutamate, respectively. The strategy has been applied to the assurance of DBS finger-prick tests from 47 pediatric patients and results affirmed with concentrations estimated in matched RBC tests using conventional HPLC-UV technique. The strategy has a potential for application in a range of clinical examinations (e.g. pharmacokinetic evaluations or medication adherence assessment) since it is minimally invasive and simple to perform, possibly allowing parents to take blood samples at home. The feasibility of utilizing DBS testing can be of significant value for future clinical trials or clinical consideration in pediatric rheumatology [25].

Mass Spectrometry

This proposed a method to determine MTX and MTXPGs in erythrocyte lysates by ultrafast Matrix-Assisted Laser Desorption/Ionization (MALDI) and triple quadrupole (tandem) Mass Spectrometry (MALDI-QqQ-MS/MS). The procedure involved SPE of MTX and MTX-MTXPG metabolites from deproteinized erythrocyte lysates using aminopterin as the internal standard. The LLOQ and LOD were 10 nmol/L and 0.3 nmol/L, respectively. The method was used to find MTX and MTX-MTXPG metabolites concentrations in patients with RA who had received low-dose oral MTX therapy [26]. The authors declared that elimination of LC in combination with MALDI, reduced analysis time therefore, this method is applicable for high-throughput measurements of large number of samples. MTXPG5 was detected as the highest glutamylation. MTXPG6 and MTXPG7 were not identified in the erythrocyte pellets of RA patients [27].

Capillary Electrophoresis (CE)

CE is applied to separate charged analytes and is based on the difference in electrophoretic mobilities of ions, leading to various migration rates. Direct injection of biological fluids to CE is possible when the drug concentration is at amounts higher than mg/L or g/L. Due to the lower amount of sample volume and smaller cell path detection in CE compared with HPLC, CE equipped with UV detector is not sensitive enough to measure drugs in mg scale. Therefore, increasing the amount of injected samples to capillary column and modifying the detector sensitivity are applied to enhance the sensitivity of CE [28].

CE utilizing less measure of sample, cheap vessels, rapid speed and high resolution and efficiency has been proposed as another procedure to isolate MTX in various matrices. Due to the significance of Cerebrospinal Fluid (CSF) during chemotherapy and relapse, the evaluation of MTX and its metabolites in CSF

was performed by triple-stacking CE. In this method, due to the low charge of sample ions, samples were injected hydro dynamically. UV-visible detection is a common detector in CE; however, it is not suitable for identification of enantiomeric impurities in biomedical samples. Therefore, Electrokinetic Chromatography (EKC) as a type of CE with luminescence detection was used to separate chiral MTX. In addition, amperometric biosensors, voltammetric methods, potentiometric membrane electrodes, TLC, HPLC and CE were also reported to be used for chiral determination of MTX.

Electrochemical Methods

Among the analytical techniques for quantitation of MTX, electrochemical methods could be an appropriate choice due to simple, inexpensive and fast procedure without having tedious sample preparation. Reduction reaction of MTX in neutral and acidic solutions is a three-step process with two-electron/two-proton transmissions. In the first step, 5,8-dihydro-MTX is formed, which tautomerizes to 7,8-dihydro-MTX. The C (9)-N (10) bond of 7,8-dihydro-MTX is separated in the next step and finally, petridine is converted into its 5,6,7, 8-tetrahydro derivative. Reduction of MTX in alkaline solutions is a single step with two-electron/two-proton transmissions, which is the result of a very slow step, so to create the reactant for subsequent reductions. Hanging Mercury Drop Electrode (HMDE) or Static Mercury Drop Electrode (SMDE) with Cyclic Voltammetry (CV) and Differential Pulse Voltammetry (DPV) or Adsorptive Stripping Differential Pulse Voltammetry (AdSDPV) has been used for the analysis of MTX by electrochemical methods. Recently, modified electrodes with nanoparticles which reduce the over potential and increase the reaction rate of many electroactive substrates have been used by different researchers [29].

Carbon nanotubes, gold and silver nanoparticles have created promising horizons for sensing systems [30]. Modification of a Glassy Carbon Electrode (GCE) with multiwalled carbon nanotubes functionalized with quaternary amine (q-MWCNTs), modification of GCE with MWCNTs immobilized within adihexadecylhydrogenphosphate film as a nanostructured patent modification of GCE with DNA functionalized Single-Walled Carbon Nanotube (DNA/SWCNT) and nation composite film for MTX quantification have been reported in recent studies. Detection limits at nanomolar (nM) range were obtained in some of the investigations [31]. For example, modification of GCE with $CoFe_2O_4$/reduced Graphene Oxide (rGO) and ionic liquid in Phosphate Buffer Solution (PBS) at pH 2.5, modification of GCE with poly l-lysine in the presence of Sodiumdodecyl Benzene Sulfonate (SDBS), AdSDPV technique along with a Bismuth Film Electrode (BiFE), synthesized nanocomposites of N-graphene coated with a bimetallic palladIum-Silver alloy (PdAg/NG-GCE), 3D porous Graphene-Carbon Nanotube (G-CNT) network on the surface of GCE, modification of GCE with cyclodextrin-graphene hybrid nanosheets achieved LODs in the range of 0.01 nm -70 nm [32].

CONCLUSION

This review examined the analysis of MTX and its metabolites from 2008 up to 2019. Literature review revealed that different analytical techniques such as HPLC using different detector systems, UV-visible Spectrophotometry, electrochemical methods and capillary electrophoresis have been employed to determine MTX. As demonstrated, LC-MS, with good selectivity and low LOQ, is the most frequently used technique for MTX analysis. HPLC was found to be an accurate method for MTX measurement. Due to polarity of the molecules and the relatively low water solubility, the reported methods for the assay of this drug are mostly reversed-phase HPLC methods using C18 silica stationary phases and mixtures of polar solvents as mobile phases. Electrochemical techniques, with low LOD, as a simple, selective and sensitive method, have been used to measure Methotrexate. However, the instrumentation and reagents are costly, hence not available in all clinical laboratories. Besides, HPLC is time-consuming and labor intensive and thus it is less commonly employed in clinical settings. Because of such analytical limitations, alternative techniques to analyze MTX are warranted. These all analytical methods are validated according to ICH guidelines.

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