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Original Article

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF METHOTREXATE, DEXAMETHASONE AND INDOMETHACIN

NARENDRA K. LARIYA*, G. P. AGRAWAL

Department of Pharmaceutical Sciences, Dr. H. S. Gour University, Sagar, M. P., India
Email: narendralariya@gmail.com

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ABSTRACT

Objective: To develop a reverse-phase high performance liquid chromatographic method for simultaneous estimation of methotrexate (MTX), dexamethasone (DXM) and indomethacin (IND).

Methods: A simple, isocratic reverse-phase high performance liquid chromatographic (RP-HPLC) method has been developed for simultaneous estimation of methotrexate, dexamethasone and indomethacin using ibuprofen (IBP) as internal standard with photodiode array detection at 254 nm. The process was carried out on C_{18} column (5 µm, 250 mm X 4.6 mm) using methanol: orthophosphoric acid (1.67 % v/v) in 70: 30 ratio at a flow rate of 1.5 ml per minute.

Results: The retention time for MTX, DXM, IND and IBP were found to be 1.47, 4.05, 11.29 and 12.69 minutes, respectively. Calibration curves of the drugs were linear in the concentration range 1-500 µg/ml. The limit of detection and limit of quantitation was 3.3 ng and 10.9 ng for MTX, 0.3 and 0.9 ng for DXM and 2.1 and 6.7 ng for IND respectively. The intra-day precision varied from 0.2 to 1.6 % and inter-day precision ranged from 0.2 to 1.8 %. The intra-day accuracy ranged from 98.3 to 101.13 % while inter-day accuracy varied from 98.6 to 101.4 %.

Conclusion: The developed method is ideally suited for simultaneous estimation of the drugs.

Keywords: RP-HPLC, Methotrexate, Dexamethasone, Indomethacin, LOD, LOQ.

INTRODUCTION

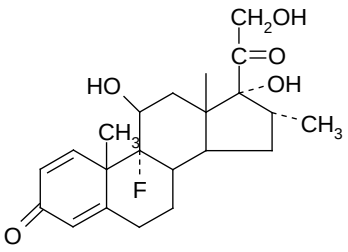
Rheumatoid arthritis is a chronic, systemic, inflammatory disorder of unknown etiology that primarily involves the joints but can also cause multiple extra-articular manifestations. Therapy has been targeted towards the treatment of the signs and symptoms of the disease as well as towards changing its natural history. Without disease modifying therapy patients with this disease neither enjoy an adequate health-related quality of life nor a normal life span. The drugs used either singly or in combination with each other include: Non-steroidal anti inflammatory drugs (NSAIDs), disease modifying anti-rheumatic drugs (DMARDs) and biologics/anticytokines. Combinations of agents from different classes are frequently employed in treatment [1].

Indomethacin, chemically 1-(4-chlorbenzoyl)-5-methoxy-2-methylindoleacetic acid, is a NSAID analgetic and antipyretic drug. Its effect is based on inhibition of cyclo-oxygenase (COX). It is frequently used for the treatment of symptoms of rheumatoid arthritis. In veterinary medicine, it is effective in treatment of inflammatory processes related to infectious diseases. The drug is usually administered orally but can also be administered intravenously or as a suppository and topical gel [2, 3].

Dexamethasone chemically is 9-fluoro-11β, 17, 21-trihydroxy-16α-methylpregna-1, 4-diene, 3, 20-dione. It is a synthetic adrenocortical steroid used to treat many different conditions such as allergic disorders, skin conditions, ulcerative colitis, arthritis, lupus, psoriasis, or breathing disorders [2, 3].

Chemically methotrexate is N- [4 (2, 4-diamino-6-pteridinyl) methyl] methyl-amino]benzoyl]-L-glutamic acid. It is used to treat rheumatoid arthritis as well as other rheumatic conditions such as juvenile arthritis, lupus, (also known as SLE), psoriatic arthritis and olymyositis (muscle inflammation) [2, 3].

Although there are number of HPLC methods for the determination of indomethacin [4-10], dexamethasone [10-16] and methotrexate [17-21] separately but to the best of our knowledge there is no HPLC method capable of determining simultaneously methotrexate, indomethacin and dexamethasone. Therefore, it was proposed to develop a method for the analysis of these drugs which are used in combination therapy.



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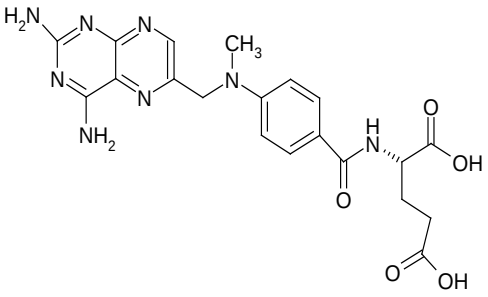

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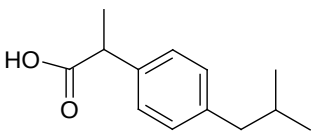

Ibuprofen

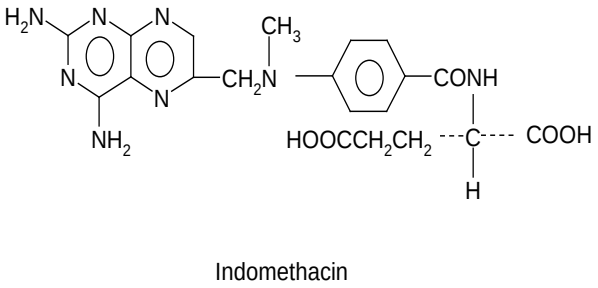

Fig. 1: Chemical structures of drugs used in the study

MATERIALS AND METHODS

Methotrexate (Dabur, Ghaziabad, India), Indomethacin (Signa Pharma, Kanpur, India), Dexamethasone and Ibuprofen (Solicito Pharma, Sagar, India) were obtained as generous gift samples. Orthophosphoric acid was purchased from Merck (Mumbai, India), methanol and water of HPLC grade were purchased from Spectrochem (Mumbai, India), membrane filters 0.45 µm (Millipore, Bangalore, India) were used for the study.

HPLC system

The HPLC system used consisted of pump SHIMADZU, LC 2O AT prominence liquid chromatography with universal loop injector Rheodyne 7725 i of injection capacity 20 uL coupled with a prominence photodiode array detector SPD M20A (Shimadzu Corporation Kyoto, Japan). The column used was Luna C18 (2) 100 R (5 µm, 250 mm X 4.6 mm) (Phenomenex, UK).

Chromatographic conditions

The mobile phase used consisted of methanol: orthophosphoric acid (1.67% v/v in water) in the ratio 70:30. The mobile phase was filtered through 0.45 µm filter in a vacuum filtration assembly before use. The eluent was monitored with a UV-visible detector set at 254 nm with the pump flow rate set at 1.5 ml/min.

Preparation of stock and working standard solutions

Stock solutions of MTX, DXM, IND (1000 µg/ml of each) and the internal standard IBP (100 µg/ml) were prepared in mobile phase. The solutions were stored at 4°C and showed no significant alterations in peak areas determined daily by direct injection throughout the study. Working standard solutions were prepared from stock solutions with an appropriate amount of drugs and internal standard stock solution to obtain the final concentration ranging from 1-500 µg/ml of methotrexate, dexamethasone, indomethacin each, and 50 µg/ml of internal standard IBP.

Assay validation [22-24]

Intra-day (within a day) and inter-day (on consecutive days) precision and accuracy of the assay was determined as precision (CV %) and accuracy (%). The samples were prepared containing 10, 25, 50, 100 µg/ml of drugs and analyzed. Three replicates of each concentration were analyzed on different days to determine inter-day precision and accuracy. Recovery studies were performed by standard addition method. To an aliquot of the analyzed sample (50 µg/ml) a known concentration of standard solutions was added (10, 25, 50 µg/ml). The contents of MTX, DXM and IND were determined. The linearity of the method was studied by analyzing the drugs at different concentrations (range 1-500 µg/ml) for MTX, DXM and IND with 50 µg/ml internal standard. Calibration data were acquired by plotting the ratio of peak area of individual drugs to that peak area of internal standard against the concentration of the calibration standard followed by a regression analysis. The constructed plots were linear and the average corresponding regression equations were y = 0.0066x+0.0252 with correlation coefficient (r^2) = 0.9995 for DXM, y = 0.0042x+0.0147 and r^2 = 0.9991 for MTX, and y = 0.0025x+0.0078 and r^2 = 0.9997 for IND, respectively where y is the ratio of peak area of drug to that of I. S. and x is the drug concentration (µg/ml). Specificity was carried out by injecting placebo solution. Limit of detection (LOD) and limit of quantitation (LOQ) were calculated using signal-to-noise (S/N) ratio method. LOD was taken as the concentration of analyte where S/N ratio was 3 and LOQ was taken as the concentration of analyte where S/N ratio was 10. The chromatographic parameters were also validated by system suitability studies, which were carried out on freshly prepared stock solutions. Ruggedness of the proposed method was determined by analysis of aliquots from homogeneous slot in different laboratories of the department, by different co-analyst, using similar operational and environmental conditions. The % CV was found to be less than 2 %.

RESULTS AND DISCUSSION

The typical chromatogram obtained from the analyses of three drugs with internal standard is presented in fig. 2. In the present analytical conditions, the retention time obtained for the drugs was 1.47 minutes for MTX, 4.05 minutes for DXM, 11.29 minutes for IND and 12.69 minutes for IBP. The calibration curve was linear in the range 1-500 µg/ml for MTX (fig. 3), DXM (fig. 4) and IND (fig. 5). The limit of detection (LOD) and limit of quantification (LOQ) for MTX, DXM and IND are reported in table 3. Intra-day and inter-day precision and accuracy of the assay is ascertained as precision (%) and accuracy (%), respectively on the basis of published guidelines [23, 24]. The obtained results are shown in table 1. The % recovery of MTX was from 99.23 to 100.69 %, for DXM was 99.2 to 100.89 % and for IND it was from 99.42 to 100.7 %. Results are shown in table 2. The results of ruggedness studies which were carried out using two similar HPLC in different laboratories by different analyst showed % CV<2 (table 4).

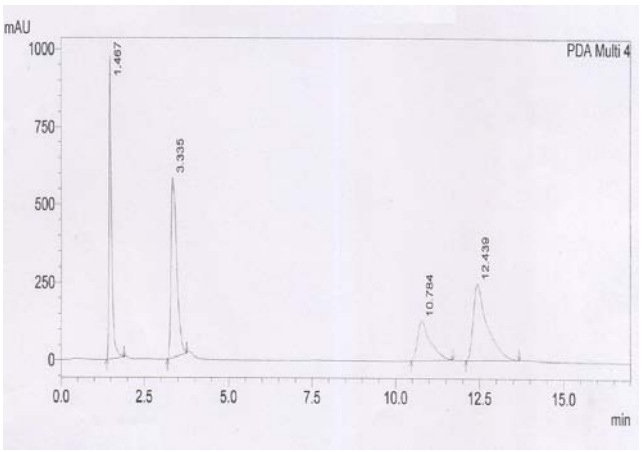

Fig. 2: Typical chromatogram obtained

Peak at 1.467 min-methotrexate, 3.335 min-dexamethasone, 10.784 min-indomethacin and at 12.439 min-ibuprofen

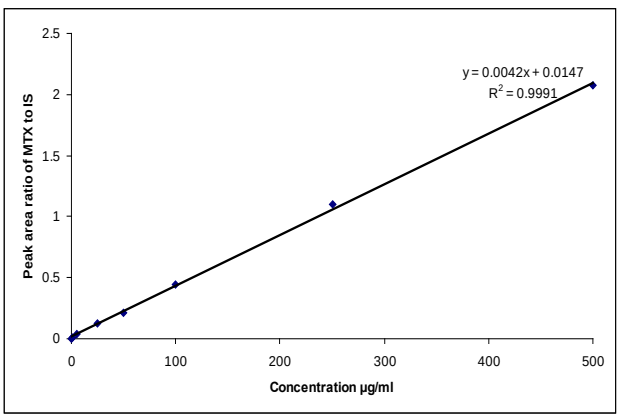

Fig. 3: Linearly regressed calibration curve of methotrexate

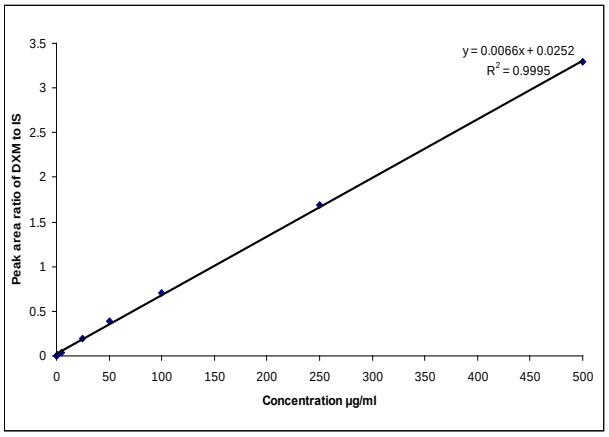

Fig. 4: Linearly regressed calibration curve of dexamethasone

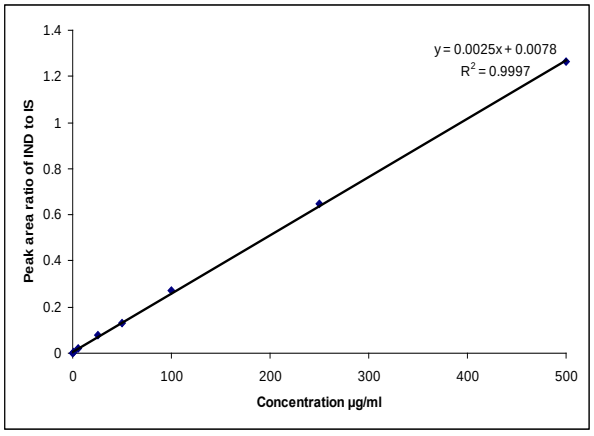

Fig. 5: Linearly regressed calibration curve of indomethacin

Table 1: Intra-day and Inter-day accuracy and precision of the assay

Drug	Conc. µg/ml	Intra-day*			Inter-day**		
		Conc. found µg/ml	Accuracy (%)	Precision (% CV)	Conc. found	Accuracy (%)	Precision (% CV)
MTX	10	10.11±.16	101.13	1.6	9.96±0.15	99.6	1.5
	25	25.13±0.25	100.52	1.0	25.35±0.45	101.4	1.8
	50	50.21±.12	100.42	0.2	50.57±.79	101.14	1.6
	100	98.36±1.29	98.36	0.3	99.99±.21	99.9	0.2
DXM	10	9.97±0.09	99.7	0.9	9.96±0.14	99.6	1.4
	25	24.94±0.14	99.76	0.5	25.13±0.31	100.52	1.2
	50	49.97±.18	99.94	0.4	49.99±.19	99.98	0.4
	100	100.1±.15	100.1	0.2	100.06±.89	100.06	0.9
IND	10	9.86±0.07	98.6	0.7	9.86±0.07	98.6	0.7
	25	25.17±0.35	100.68	1.4	25.1±0.1	100.4	0.8
	50	49.97±.16	99.94	0.3	50.37±.53	100.74	1.1
	100	99.71±.61	99.71	0.6	99.69±.56	99.69	0.6

Values = mean±SD * (n = 3), ** (n = 9)

Table 2: Percent recovery obtained by standard addition technique

Drug Conc. (50 µg/ml)	Added Conc. µg/ml	Conc. Found µg/ml	Extraction recovery (%)	CV (%)
MTX	10	60.40±0.6	100.69±1.03	1.0
	25	75.27±0.5	100.35±0.68	0.7
	50	99.23±0.85	99.23±0.85	0.9
DXM	10	60.37±0.73	100.61±1.22	1.2
	25	75.67±0.87	100.89±1.17	1.2
	50	99.2±1.05	99.2±1.05	1.1
IND	10	59.96±0.89	99.93±1.48	1.5
	25	74.57±0.82	99.42±3.3	1.09
	50	100.7±1.2	100.7±1.2	1.1

Values = mean±SD (n = 3)

Table 3: System suitability parameters

Drugs	Methotrexate	Dexamethasone	Indomethacin
Calibration range µg/ml	1-500	1-500	1-500
Retention time (min.)	1.47	4.05	11.29
HETP	111.46	53.845	33.354
Tailing factor	1.54	1.32	1.35
Theoretical plates	2071	2785	4497
LOD (ng)	3.3	0.3	2.1
LOQ (ng)	10.9	0.9	6.7
Resolution factor	11.163	14.834	
K'	1.788	6.810	

Table 4: Results of ruggedness study

Samples	Drugs	Analyst 1 (µg/ml)	Analyst 2 (µg/ml)	% CV
Sample I	MTX	5.1±0.78	5.2±0.97	1.4
	DXM	5.06±0.59	5.2±0.86	1.9
	IND	5.2±1.02	5.29±.93	1.2
Sample II	MTX	10.14±1.26	9.9±1.12	1.7
	DXM	10.2±0.84	10.03±0.74	1.2
	IND	9.88±0.47	10.02±0.38	1.0
Sample III	MTX	24.97±0.27	25.65±0.41	1.9
	DXM	25.66±0.43	25.34±0.58	0.9
	IND	24.78±0.82	25.41±0.27	1.8
Sample IV	MTX	49.78±0.76	50.16±0.36	0.5
	DXM	50.78±0.69	51.25±0.34	0.7
	IND	49.76±1.04	50.46±0.57	1.0
Sample V	MTX	100.46±0.74	101.32±0.15	0.6
	DXM	100.96±0.25	99.82±0.34	0.8
	IND	99.54±0.39	99.04±0.24	0.4

Values = mean±SD (n = 3)

CONCLUSION

A simple, sensitive, economic and reliable RP-HPLC method has been developed and validated for simultaneous estimation of methotrexate, dexamethasone and indomethacin using ibuprofen as an internal standard. The method is accurate, rapid, reproducible, specific and rugged for simultaneous determination of these drugs.

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CONFLICT OF INTERESTS

Declared None

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