Research Article Open Access

ARTICLE LINK

ISSN: 2349-9818

JULY – AUGUST 2021, VOL. 8 (4), 01-10

TROPICAL JOURNAL OF PHARMACEUTICAL AND LIFE SCIENCES

(An International Peer Reviewed Journal)

Journal homepage: http://informativejournals.com/journal/index.php/tjpls

DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF CLARITHROMYCIN, TINIDAZOLE AND LANSOPRAZOLE IN FIXED DOSE COMBINATIONS

Basant Lal[1]*, Manish Jaimini[1] and Devesh U Kapoor[2]

[1]Department of Pharmaceutical Sciences, Maharishi Arvind University, Mundiaramsar, Jaipur-302012, (Raj.), India

[2]Department of Pharmacy, Dr. Dayaram Patel Pharmacy College, Bardoli, Gujarat-394601, India

ARTICLE INFO:

Received: 11th July 2021; **Received** in revised form: 14th August 2021; **Accepted:** 25th August 2021; **Available online:** 30th August 2021.

ABSTRACT

New RP-HPLC method was developed for the simultaneous estimation of clarithromycin, tinidazole and lansoprazole in the fixed dose combination (PYLOKIT by CIPLA). RP-HPLC separation was carried out using Ultisil XB-CN column (250 x 4.6 mm) internal diameter and the packing material having 5µm size using gradient mobile phase of Potassium dihydrogen phosphate: acetonitrile. 1 mL/min was the flow rate and UV detector at 210 nm wavelength was fixed for detection of the drug. The method validation was done as per ICH guideline and the parameters were included such as accuracy, precision specificity, linearity, and robustness were determined. Retention times for the clarithromycin, tinidazole and lansoprazole were 16.643, 5.493 and 12.940 minutes respectively. RP-HPLC method was a simple, reliable and acceptable and it confirmed that method is suitable for the intended use for routine quality control and assay of drugs. This method is successfully applied for the determination of commercial dosage form preparation. This method is validated as per ICH (International conference on harmonization) guidelines.

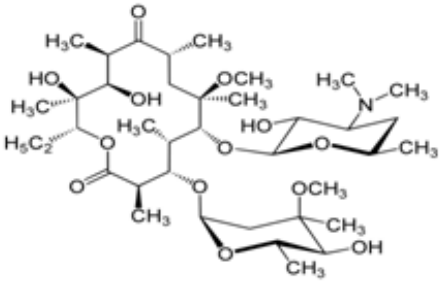
Keywords: RP-HPLC, Clarithromycin, Tinidazole, Lansoprazole, Method development, Method validation.

INTRODUCTION

Peptic ulcers are localized erosions of the mucous membranes of the stomach and duodenum. The pain associated with ulcers is caused by irritation of exposed surfaces by the stomach acids. The current approach for treating ulcers caused by *Helicobacter pylori* is to use combination of drugs, which includes a proton pump inhibitor and two antimicrobials, such as tinidazole and amoxicillin or clarithromycin.

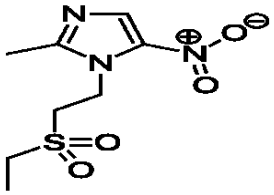
***Corresponding Author:**
Basant Lal,
Department of Pharmaceutical Science,
Maharishi Arvind University,
Mundiaramsar, Jaipur-302012,
(Raj.), India

Structure of Clarithromycin



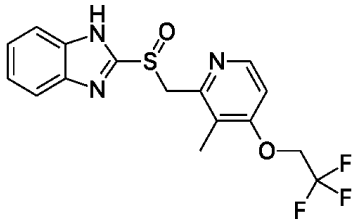
Clarithromycin is a semi-synthetic macrolide antibiotic derived from erythromycin A. It consists of a 14 membered lactone ring as well as cladinose and desosamine residues at positions 3 and 5 of the ring, respectively. Like erythromycin, it has no conjugated double bond in the lactone ring, hence significant UV absorbance is only obtained at wavelengths below 210 nm.[1,2] It is white or almost white crystalline powder, practically insoluble in water, slightly soluble in alcohol and acetonitrile and freely soluble in acetone. It may be bacteriostatic or bactericidal depending on the organism and drug concentration and it mainly acts by inhibiting bacterial protein synthesis. The drug is used to treat pharyngitis, tonsillitis, acute maxillary sinusitis, acute bacterial exacerbation of chronic bronchitis, pneumonia, skin infections, etc.[3] Literature survey revealed that various methods have been developed for the estimation of clarithromycin from laboratory prepared mixtures, pharmaceutical preparations and biological matrices (such as human plasma) through automated solid phase extraction and electrochemical detection, liquid chromatographic electrospray tandem mass spectrometry and high performance liquid chromatography.[4-7]

Structure of Tinidazole



Tinidazole has activity against anaerobic bacteria and protozoa. It is used to eradicate *H. pylori* in peptic ulcer disease with other antimicrobials and proton pump inhibitor[8]. It had been determined spectrophotometrically[9-11] and through HPLC.[12-14]

Structure of Lansoprazole



Lansoprazole, chemically known as 2-[[3-methyl- 4-(2,2,2-trifluoroethoxy) pyridin-2-yl] methylsulfinyl] -1H-benzimidazole. (Molecular Formula: $C_{16}H_{14}F_3N_3O_2S$, Molecular weight: 369.36, CAS no 103577-45-3). Lansoprazole, a member of the proton-pump-inhibitor class of gastric acid inhibitory agent, effectively raises intragastric pH and is indicated for the short-term treatment of active erosive reflux esophagitis, gastric ulcer, duodenal ulcer, and nonerosive gastroesophageal reflux disease. As a proton-pump inhibitor, lansoprazole is also a necessary component of dual- and triple therapy regimens for the eradication of *Helicobacter pylori* infection. The latest FDA-approved labeling for lansoprazole includes the indication of healing and risk reduction in nonsteroidal anti-inflammatory drug-associated gastric ulcers.[15-17] Literature survey revealed that various methods have been developed for the estimation of Lansoprazole.[18-21]

Till date only one RP-HPLC method is reported by Dadhich *et al.*[22] for the simultaneous estimation of the three drugs which is in isocratic mode. So, it is the need to develop a simple and gradient programming RP-HPLC to estimate the content of the three drugs in the tablet/capsule formulation.

MATERIALS AND METHODS

Clarithromycin, tinidazole and lansoprazole were the gifted samples obtained from Uttranchal Research and Testing laboratory, Uttrakhand. The Samples were characterized by Infrared Spectroscopy (IR) and Mass Spectroscopy for checking the purity level.

RANKEM Chemicals supplied the AR grade chemicals of potassium dihydrogenphosphate, orthophosphoric acid (OPA), methanol and HPLC grade acetonitrile. Milli-Q water purification system produced water was used during analysis (Make & Model: MILLIPORE/Integral 5).

Instrumentation and Software

The validated analytical method was performed on HPLC (Make & Model: Agilent with UV Detector) which is equipped with solvent delivery pump, degasser, using Openlab® Software.

RP-HPLC separation was carried out using Ultisil XB-CN column (250 x 4.6 mm internal diameter and the packing material having 5µm size) using gradient programming mobile phase of buffer: acetonitrile (The pH was adjusted to 4.0 by the help of orthophosphoric acid). 1 mL/min was the flow rate and UV detector at 210 nm wavelength was fixed for detection of the drugs.

Methods

<table>
<tr><th colspan="2">Chromatographic conditions</th></tr>
<tr><td>Column</td><td>Ultisil XB-CN, column (250mmx4.6mm), 5 µm</td></tr>
<tr><td>Mobile phase</td><td>Gradient programming
<table>
<tr><th>Time</th><th>Buffer (KH_2PO_4)</th><th>Acetonitrile</th></tr>
<tr><td>0</td><td>90</td><td>10</td></tr>
<tr><td>17</td><td>70</td><td>30</td></tr>
<tr><td>20</td><td>70</td><td>30</td></tr>
<tr><td>21</td><td>90</td><td>10</td></tr>
<tr><td>25</td><td>90</td><td>10</td></tr>
</table></td></tr>
<tr><td>Detector</td><td>UV detector</td></tr>
<tr><td>Flow rate</td><td>1 ml/min</td></tr>
<tr><td>Wavelength</td><td>210nm</td></tr>
<tr><td>Injection Volume</td><td>10µL</td></tr>
<tr><td>Temperature</td><td>30°C</td></tr>
<tr><td>Diluent</td><td>Methanol</td></tr>
</table>

Preparation of Standard Solution

The stock solutions were prepared by dissolving 37mg of lansoprazole in 25 ml flask, add approx. 10 ml of diluent and mix well. Make the volume. Take 1 ml of solution from this solution in 100 ml flask. Add 12.5 mg of clarithromycin and 25 mg of tinidazole. Mix well with diluent and finally make the volume to prepare concentrations of 125 µg/mL for clarithromycin, 250 µg/mL for tinidazole and 15 µg/mL for lansoprazole respectively.

Preparation of Sample Solution

The sample solutions were prepared by dissolving 1/10 of average weight of drugs in 100 ml flask and mix well with diluents to prepare concentrations of 125 µg/mL for clarithromycin, 250 µg/mL for tinidazole and 15 µg/mL for lansoprazole respectively.

Method Validation

The developed method was validated with respect to system suitability, specificity, linearity, precision, accuracy LOD, LOQ and robustness in the accordance of the ICH Q2 guidelines.

Specificity and Selectivity

The developed method was found to be selective for clarithromycin, tinidazole and lansoprazole, since the injection of the blank solution confirmed the absence of interfering peak at RT examined substance at 210nm.The results obtained demonstrate that there was no interference from other material in the developed method and therefore confirm the specificity of the method.

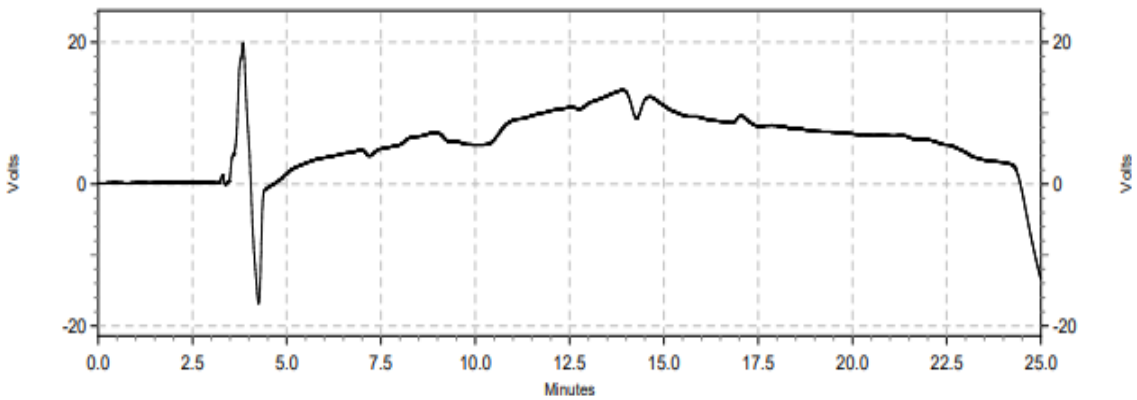
System Suitability

System Suitability tests are an integral part of method development and were used to ensure adequate performance of the chromatographic system. Retention Time (RT), tailing factor, peak asymmetry, and theoretical plates (T) were evaluated. The results are shown here in Table 1.

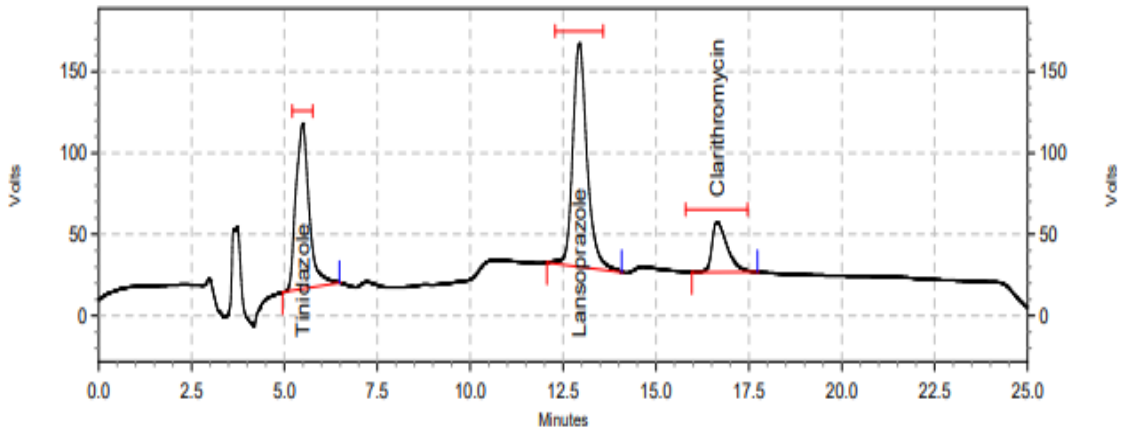
Table 1: **System suitability parameters of Clarithromycin, tinidazole and lansoprazole**

Sr. No.	Property	Clarithromycin	Tinidazole	Lansoprazole	Acceptance criteria
1.	Retention Time (RT)	16.643	5.493	12.940	-
2.	Tailing factor (T)	1.21	1.14	1.32	NMT 2.0
3.	Theoretical plates (N)	4136	5264	3638	NLT 2000

From the data it was found that all the system suitability parameters for developed method were within the limit.



Chromatogram: Blank



Chromatogram: Standard

Linearity and Range

Linearity of the developed method demonstrates the ability of method to produce a result which is directly proportional to concentration of analyte in the sample. The amount of clarithromycin, tinidazole and lansoprazole were prepared for linearity in the range of 80-120%. The amount of clarithromycin, tinidazole and lansoprazole in five different concentrations is 80%, 90%, 100%, 110% and 120% of working strength respectively. The graph was plotted between concentrations versus area of peak. The clarithromycin, tinidazole and lansoprazole shows good correlation coefficients (R^2= 0.9991, 0.9989 and 0.9994) and the proposed method was linear in concentration range 80-120 %.

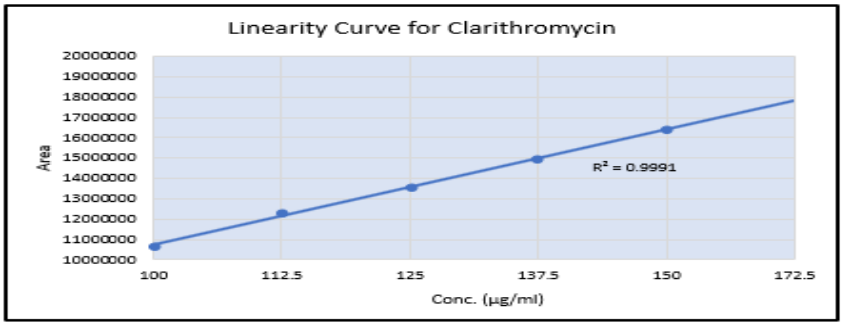
Table 2: **Linearity of Clarithromycin, Tinidazole and Lansoprazole**

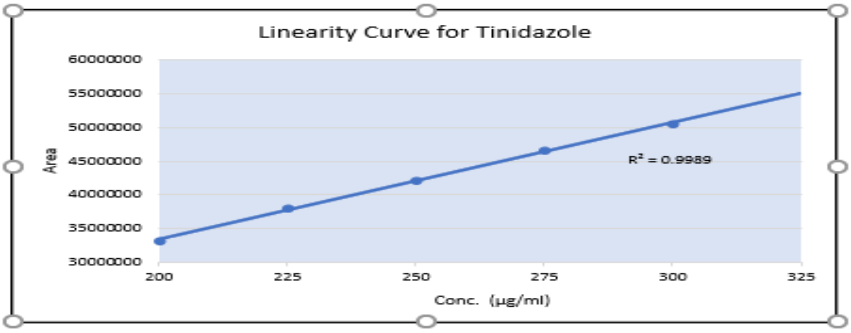
S. No.	Compound	Values of X and Y Variables						Correlation co-efficient
1	Clarithromycin	Variable	1	2	3	4	5	0.9991
		X	100	112.5	125	137.5	150	
		Y	10652847	12258564	12258564	12258564	12258564	
2	Tinidazole	X	200	225	250	275	300	0.9989
		Y	33126545	37994628	42137214	46575128	50536299	
3	Lansoprazole	X	12	13.5	15	16.5	18	0.9994
		Y	48462163	54905648	61492166	67572584	73398547	

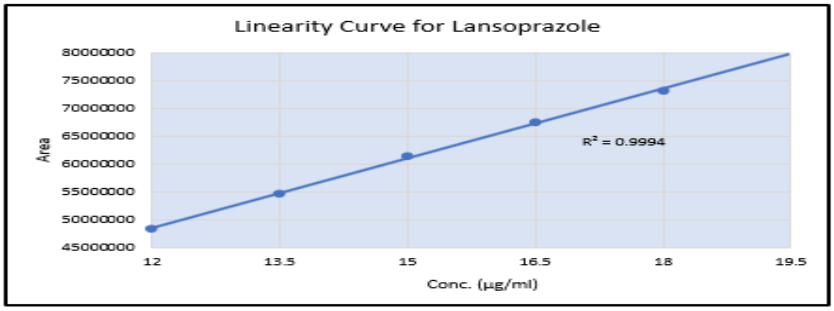
Note: X is the concentration of the respective component in µg/mL. Y is the peak response of the respective component in area counts.

Linearity Curve

Calibration curve was constructed between concentrations versus peak area. Results were recorded for equation of line and correlation co-efficient were determined.







Precision

It reveals the data regarding closeness between the series of measurements. The precision of the developed method was verified by system precision and method precision. A homogenous sample concentration of 125 µg/mL for clarithromycin, 250 µg/mL for tinidazole and 15 µg/mL for lansoprazole respectively were prepared under prescribed conditions and estimation was carried out. The results are expressed in the form of standard deviation and RSD value. Table 3 and 4 shows the result of system precision and method precision respectively and the developed method is highly precise as % RSD is less than 2%.

Table 3: **Calculation of %RSD for Clarithromycin, tinidazole and Lansoprazole (System Precision)**

S. No.	Compound	No. of Injections						Mean	S.D.	%RSD
		1	2	3	4	5	6			
1	Reference Standard Clarithromycin	14852634	14874629	14812463	14884614	14901663	14863265	14864878	30781.7396	0.207
2	Reference Standard Tinidazole	42153629	42130524	42162051	42201464	42231451	42185247	42177394.33	36222.0196	0.086
3	Reference Standard Lansoprazole	61732184	61751477	61721465	61804715	61825695	61774188	61768287.33	41075.0577	0.066

Table 4: **Calculation of %RSD for Clarithromycin, tinidazole and Lansoprazole (Method Precision)**

S. No.	Compound	No. of Injections						Mean	S.D.	%RSD
		1	2	3	4	5	6			
1	Sample Clarithromycin	13771885	13740332	13732656	13763211	13784155	13780817	13762176	21328.3378	0.155
2	Sample Tinidazole	41372144	41350207	41330962	41353298	41385277	41383607	41362582.5	21390.1995	0.052
3	Sample Lansoprazole	61432166	61419568	61401822	61428558	61452148	61458954	61432202.67	21020.7269	0.034

Mean represents the average values of six replicates analysis. **SD** is the standard deviation calculated on the six replicates. **RSD** is the relative standard deviation.

Table 5: System Precision and Method precision

Precision	Drug	% RSD
System precision	Clarithromycin	0.207
Method precision	Clarithromycin	0.155
System precision	Tinidazole	0.086
Method precision	Tinidazole	0.052
System precision	Lansoprazole	0.066
Method precision	Lansoprazole	0.034

Accuracy

It is also termed as trueness or recovery. This method was determined using 80%, 100% and 120% of working strength of Clarithromycin, tinidazole and lansoprazole. Each level solution was prepared in duplicate and analysed as per the method given. This is usually demonstrated in the form of SD and RSD. The results reveal that the value of % RSD is less than 2%. The percent recovery results are in Table: 6.

Table 6: Summary of assay of Clarithromycin, tinidazole and lansoprazole

S. No.	Level	Compound	% Average Assay	%RSD
1	80%	Clarithromycin	98.49	0.26
		Tinidazole	99.53	0.17
		Lansoprazole	99.69	0.18
2	100%	Clarithromycin	98.96	0.23
		Tinidazole	99.20	0.20
		Lansoprazole	99.76	0.11
3	120%	Clarithromycin	98.94	0.24
		Tinidazole	99.21	0.18
		Lansoprazole	99.88	0.13

The percentage of assay values of clarithromycin were in the range of 99.49-99.96 %, tinidazole in the range of 99.20-99.71 % and lansoprazole in the range of 99.53-99.88%. The % RSD of assay values of clarithromycin were in the range of 0.21-0.26 %, tinidazole in the range of 0.16-0.26 % and lansoprazole in the range of 0.13-0.18%. The study proves that the method is accurate for the estimation of amoxicillin, tinidazole and omeprazole assay over the range of 80-120% of target concentration.

LOD and LOQ (Limit of Detection and Limit of Quantification)

Limit of detection (LOD) and Limit of Quantification (LOQ) reveal information regarding concentration of analyte that yields signal-to-noise around 1 to 10. Serial dilutions are made from solution of clarithromycin, tinidazole and lansoprazole for determination of LOQ and LOD. The samples were injected in HPLC and compare the signals of sample and blank sample of LOD and LOQ. According to earlier mentioned parameters, LOD and LOQ were estimated for clarithromycin, tinidazole and lansoprazole were 2.5 µg/ml, 5 µg/ml and 0.5 µg/ml and 7.5 µg/ml, 15µg/ml and 1.5 µg/ml respectively.

Robustness

The robustness of the developed HPLC method was carried out by making small deliberate changes in the HPLC process parameters. These parameters include variation in wavelength, flow rate of mobile phase and changes in proportion of buffer and acetonitrile. The method was performed on single concentrations of clarithromycin, tinidazole and lansoprazole. The alteration of parameters may leads to some significant changes in the peak area and RSD. Robustness studies concludes that the method is robust under ± 2 wavelength, ± 10% flow rate and ± 10% increase and decrease in mobile phase and at the different column (Zorbax CN column (250mmx4.6mm), 5 micron. There is no significant change in recovery of amoxicillin, tinidazole and omeprazole. The % RSD shown in table:7 negligible changes were observed during robust condition. So, we can say that the developed method is robust.

Table 7: Robustness Data

Drug	Parameters	% RSD
Clarithromycin, tinidazole and Lansoprazole	Wavelength minus	0.004
	Wavelength plus	0.003
	Flow minus	0.002
	Flow plus	0.004
	Mobile phase ratio change	0.002
	Column Change	0.001
	Temperature minus	0.005
	Temperature plus	0.004

RESULT AND DISCUSSION

After a number of trials with different, mobile phases were tested but the adequate separation of Clarithromycin, tinidazole and lansoprazole was found in Potassium dihydrogen phosphate: Acetonitrile (Gradient programming). The best results were obtained with flow rate gradient programming of selected mobile phase for the purpose of rapid analysis. Mobile phase was started at a flow rate of 1.0 ml/min which was continued for 1.0 min to 25.00 min.

The validation of the developed and the optimized RP-HPLC method was carried out with respect to the parameters such as specificity, linearity, accuracy, precision, limit of quantification (LOQ) and limit of detection (LOD) in the light of internationally accepted ICH guidelines.

CONCLUSION

The HPLC method was successfully developed and validated on an Agilent 1220 LC for simultaneous determination of clarithromycin, tinidazole and lansoprazole in PYLOKIT combination. This present method is simple and accurate for the determination of drug at a single wavelength, 10 µL injection capacity and Ultisil XB C18 5µm 4.6*150 mm column. It was found that the method is sufficiently simple, rapid and sensitive as well as precise, accurate, linear, robust which compiles the ICH guidelines. The

entire experimentation was proved that the developed HPLC method shows good resolution, linearity and RSD values (less than 2%) which indicates that method is suitable for the estimation of clarithromycin, tinidazole and lansoprazole.

ACKNOWLEDGEMENT

Authors are thankful to Uttrakhand Research and Testing Laboratory, Uttrakhand for the necessary instruments and facility provided.

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How to cite this article: Basant Lal, Manish Jaimini and Devesh U Kapoor (2021), "DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF CLARITHROMYCIN, TINIDAZOLE AND LANSOPRAZOLE IN FIXED DOSE COMBINATIONS", ***Tropical Journal of Pharmaceutical and Life Sciences (TJPLS Journal)***, 8(4), 01-10. Retrieved from https://informativejournals.com/journal/index.php/tjpls/article/view/69

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