International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304, ISSN(Online): 2455-9563
Vol.15, No.02, pp 28-38, 2022

TO Develop Method on RP-HPLC and Validate for the Determination of Plasticiser (Di-Octyl Phthalate) Content in Re-constituting Diluents and Re-constituted Solutions of Ciprofloxacin Injection

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Abstract : A simple, rapid, accurate and economic reverse phase HPLC method was developed and validated for determination of di-(2-ethylhexyl)phthalate (DOP) in re-constituting diluents and reconstituted solutions of Ciprofloxacin for injection The method was applied to detect any leaching enhancement in presence of Ciprofloxacin reconstituted injections. Ciprofloxacin for injection is a widely used broad spectrum beta-lactam antibiotic. As per requirements of various regulatory authorities of different countries, DOP content needs to be monitored in the re-constituting diluents used to reconstitute injections. The proposed method is a unique method wherein DOP can be determined directly without any sample pre-treatment before analysis. The method can be used as a good quality control tool to control the leaching of DOP in the re-constituting diluents and reconstituted injections**. Method:** C18 column (250 x 4.6 mm, 5µ) and a mixture of methanol, Acetonitrile and water as mobile phase. DOP was detected at 225 nm. The method has low limit of quantification (0.058 µg mL-1) which is much below the acceptance limit calculated as per USFDA tolerance criteria (3.5 µg mL^{-1} for adults and 0.3 µg mL^{-1} for neonates and infants).

IndexTerms - **HPLC, DOP, PVC, FDA, Re-constituting diluents, Ciprofloxacin for injection.**

Keywords : Desalination, Adsorption, Reverse Osmosis, Chemical Precipitation, Bio sorbent.

DOI: http://dx.doi.org/10.20902/IJPTR.2022.150202

I. Introduction

Di-Octyl Phthalate, more commonly known as DOP (CAS No. 117-81-7) is an clear liquid with mild odour. Slightly less denser and insoluble water with chemical formula $C_{24}H_{22}O_4$. It is ester of phthalic acid manufactured using phthalic anhydride and 2-ethyl hexanol as pricursor materials. It is most widely used plasticizer due to higher boiling point (385 °C) in polyvinyl chloride (PVC) providing good heat absorption capcity. It has low mol. wt. (390.56) giving good lubricancy and system floabilty which in turn provides excellent plasticity and softness to PVC[1]. Moreover, PVC being cheap and having excellent properties is one among the most commonly used plastic materials for medical and surgical devices. DOP is extensively used in manufacturing PVC medical and suregical devices like catheters, blood bags, infusion bags, feeding tubes, infusion tubings,etc. Virtually all PVC medical devices utilize DOP as plasticizer. It can leach out from PVC matrix resulting in its exposure and poisoning to body tissues and fluids [1]. Thus, there are high possibilities of DOP exposure and Poisoning during medical procedures such as blood and blood products transfusion, haemodialysis, heart bypass surgery, extracorporeal membrane oxygenation and administration of IV fluids. The highest human exposures to DOP can occur in infants and newborns undergoing extensive medical procedures which may lead to a body DOP level between 130-6000 µg kg body weight-1 day[1,2] .

DOP can be metabolized in human body rapidly by the enzymes mainly present in the intestines that break down the DOP into di-n-pentyl phthalate (DPP). This enzymatic conversion also occurs in the pancreas, kidney, liver, lungs and plasma. DOP metabolism involves a complex series of reactions that may produce 25 or even more metabolites, prominently several keto acid derivatives, 2-ethylhexanol, DPP and 2-ethylhexanoic acid that are responsible for its toxicity [3-7]. DOP also accumulates in adipose tissues [3]. It is secreted through milk in lactating females in significant amount that affects adversely the new born and infants [2].

Studies in laboratory with rodents shows DOP and its metabolites affects adversely in reproduction and produce testicular toxicity, in males underdeveloped reproductive tracts, prominently in new borns and infants [1,2,4].DOP exposure may also lead to bronco-contraction, contact urticaria syndrome, carcinogenicity, immunosuppression and mutagenicity . Testes, Liver and Kidney are the other main organs targeted by DOP induced toxicity [1].

Thus, Phthalates leached from PVC medical devices as well as Primary Packaging System contributing to body fluids and systems is strictly monitored by regulatory agencies. “USFDA” provides different tolerance limits for neonates and adults of leached DOP from various Plastic mediated medical devices. For IV infusion of drugs requiring P’ceutical vehicles to solubilize and to prior administration, the permissible daily exposure of DOP is limited to 0.04 mg kg body weight^{-1} day^{-1} for adults (75 kg) and 0.03 mg kg body weight^{-1} day^{-1} for neonates (4.5 kg) [8] .

The diluents used for re-constituting Ciprofloxacin for injection are 0.9 % Normal Saline injection, 5% Dextrose injection [9]. These re-constituting diluents are supplied in PVC bags, which may lead to DOP leaching in the reconstituents. Further, presence of Ciprofloxacin may also contribute to DOP leaching to re-constituents as stored in PVC packaging. As per the bag insert of Ciprofloxacin for injection, the re-constituted injection may be stored for 3 to 4 hrs at RT (up to 25°C) or for 24 hrs under refrigeration (below 4 °C) [9]. Hence, the concentration of DOP in re-constituents and re-constituted injections both at the initial stage; at extreme storage time points and need to be monitored by an appropriate analytical method.

A variety of analytical methods are available for determination of DOP in various biological and non biological matrix. Some methods involve preconcentration by liquid- liquid extraction of DOP to organic phase like hexane or methylene chloride or use combination of methanol and acetonitrile as eluent but these methods may not be suitable for determination of leached DOP in some re-constituting diluents (e.g. mannitol) due to precipitation of osmogens [10-11]. Other methods using hexane and ethyl acetate as mobile phase components to monitor DOP and its metabolites in plasma are cumbersome as these methods require lengthy and time consuming solvent-solvent extraction of aqueous samples before analysis [12]. A variety of sophisticated and expensive analytical methods are available that utilize techniques like LCMS, GC, GC-MS, electron capture GC13-15 or involve micro-organic ion association phase extraction [16] to determine DOP and metabolites in plasma or samples from variety of matrices. Most of the mentioned methods are not simple and cannot be used directly without prior sample treatment thus limiting their applicability.

A simple, accurate, sensitive, rapid and economic reversed phase high performance liquid chromatography (RPHPLC) method has been developed that does not involve any preconcentration in organic phases and is

capable of analyzing aqueous samples directly. This method involves very simple and fast sample preparation and takes less time for analysis since run time is only 15 minutes. Further, the method has been extended to determine the content of DOP in re-constituted Ciprofloxacin injection in various re-constituting diluents [9]. As per dosage regime of Ciprofloxacin for injection, the maximal acceptable amount of DOP in reconstituted injection is calculated to be 3.5 µg mL^{-1} for adults and 0.3 µg mL^{-1} for infants and neonates. The limit of quantification of the proposed analytical method is 0.058 µg mL^{-1} which is low enough to quantify DOP accurately in reconstituted injections much below the tolerance limits [8].

II. Experimental

HPLC Method	Specification Values
Coloumn	C_{18} (20x4.6mm)(20x2.1mm)
Moblile Phase	Acetonitrile:Methanol(9:1)
Injection Vol.	20µl
Flow Rate	0.8 ml/min(4.6mm coloumn) 0.17 ml/min(2.1mm coloumn)
Wavelength of Detection	225nm
Total Flow	1.2000ml/min
Pump [A] Pressure	141kgf/cm^2
Pump [B] Pressure	137kgf/cm^2
Pump [A] Degasser	-
Pump [B] Degasser	-
Mode	Binary Gradiant
End Time	15 min
Retention Time	8 min

To prepare mobile phase and diluent, HPLC grade methanol and acetonitrile were procured from VBCOP Chemical Store and HPLC grade water was also procured from VBCOP Chemical Store. DOP working standard (99.74% pure) was procured from Loba Chemie (Mumbai, India). The re-constituting diluents 0.9% sodium chloride, 5% dextrose injection were procured in PVC bags from Baxter (Deerfield, IL, USA). Ciprofloxacin Concentrate for injection(Ciprox) samples were procured from Claris Injectables limited (Ahmedabad, Gujrat, India).

2.1 Preparation of standard solution

About 1 g of DOP Std. was weighed accurately into a 100 mL dried and cleaned vol. flask, previously containing about 80 mL of methanol. Solution was allow to sonicated with constant swirling to mix the contents and volume was made up to the lower meniscus mark with methanol. 1 mL of this solution was transferred again into a 100 mL vol. flask and volume was made up to the mark with diluent. This solution was suitably diluted with diluent to get a final concentration of about 1 µg mL^{-1}.

2.1 Preparation of sample solution

Injection vial was reconstituted with 100 mL of re-constituting diluent to get a clear reconstituted solution and 5 mL of this was further diluted to 10 mL with diluent. During inuse stability studies, samples were stored at different temperatures and analysed at various time points. Reconstituted samples were stored in reconstituent bags as per instructions given on pack insert9 . Prior to analysis, suitability of the chromatographic system was ensured by injecting blank and standard solutions.

Blank was injected to check any interference at the retention time of DOP. System suitability parameters, USP tailing (not more than 1.5) and USP plate count (not less than 4000) were monitored for DOP peak in standard solution injections. The precision of the chromatographic system was ensured by checking the %RSD of area counts of five replicate injections of standard solution (not more than 2.0%). The proposed analytical method was validated for specificity, linearity, precision, accuracy, and stability in analytical solution as per ICH17 guidelines.

2.3 Specificity

Specificity of the method was checked by injecting the standard solution, sample solution and sample solution spiked with known related impurities of Ciprofloxacin recommended re-constituting diluents

2.4 Linearity of response and limit of quantification (LOQ)

The linearity of the method was checked in the concentration range of 0.14 to 8.83 µg mL^{-1} of DOP.

2.5 Precision

Six replicates injections of standard solution were given to establish system precision. To establish method precision, six samples of single batch of Ciprofloxacin for injection were prepared independently using 0.9% Normal Saline Solution and 5% Dextrose solution as re-constituting diluent and analyzed.

2.6 Accuracy

Known amount of sample was spiked in triplicates with known quantities of DOP at different levels using 0.9% Normal Saline and 5% dextrose as re-constituting diluents. The samples were analysed by the proposed method and amount of DOP recovered was calculated after making corrections for the amount already present.

2.7 Stability in analytical solution (SIAS)

Sample solutions prepared using the diluents 0.9% Normal Saline and 5% dextrose were injected repeatedly at various time intervals up to about 15 hrs keeping the sample solutions at room temperature during the study.

2.8 Monitoring DOP content in reconstituted Ciprofloxacinfor injections

Ciprofloxacin for injections were reconstituted separately in 0.9% Normal Saline and 5% dextrose stored in PVC bags for 24 hrs at 4 °C and for 4 hrs at 25 °C.

III. Results and Discussion

Since the DOP has chromophoric groups, attempts were made using HPLC with UV detection to develop the analytical method for determination of DOP content in aqueous samples. Initially, a chromatograph equipped with C18, 250 x 4.6 mm, 5 µm, utilizing acetonitrile and methanol combination as mobile phase was used to determine DOP in aqueous samples10. Peak shape of DOP was symmetrical for samples prepared in dextrose, sodium chloride and 5% dextrose with 0.02% sodium bicarbonate reconstituents. However, sample got precipitated when sample reconstituted using mannitol were diluted using this diluent.

To overcome precipitation issue observed, hexane extraction was tried for extraction of DOP from reconstituted samples. Again, precipitation was observed using this sample preparation technique.

Further, water and propan-2-ol were introduced in mobile phase composition along with methanol. A suitable ratio of methanol, propan-2-ol and water was optimized as eluent and diluent to obtain clear solution. Using this ratio, the sample solution remained clear when it was stored for the recommended period. The finalized method was found suitable for direct analysis of aqueous samples without any preconcentration or liquid extraction and was found applicable to all the reconstituents used for reconstitution of Ciprofloxacin for injection. The proposed method was optimized and validated as per ICH18 guidelines. Peak purity plot of DOP peak indicated the peak was pure and there were no co-eluting peaks Figure 1-6. This indicated the specificity of the developed method.

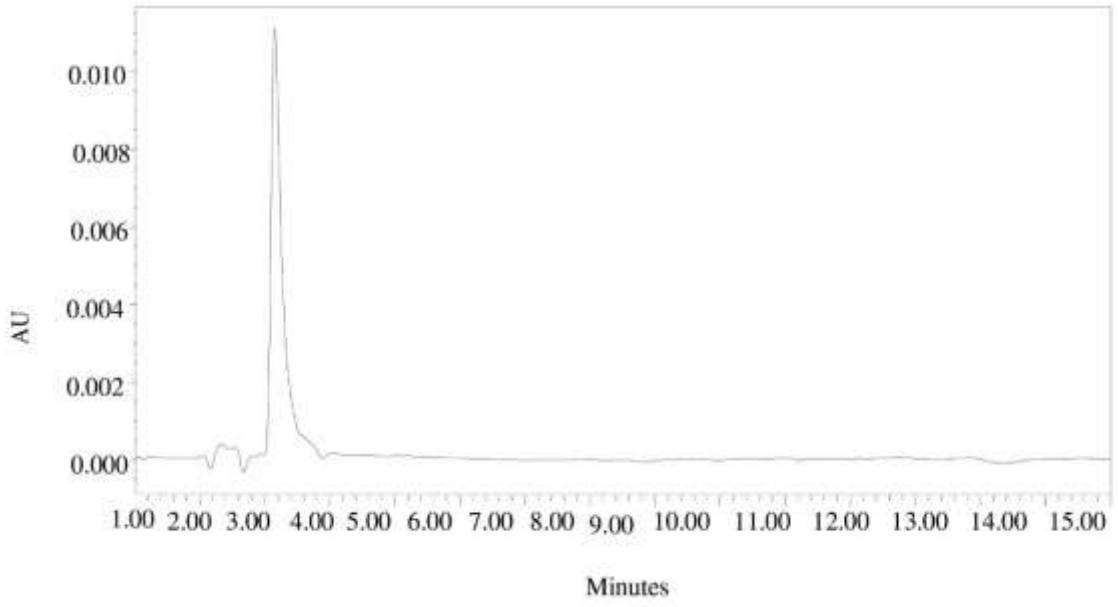


Figure 1. Chromatogram of blank solution.

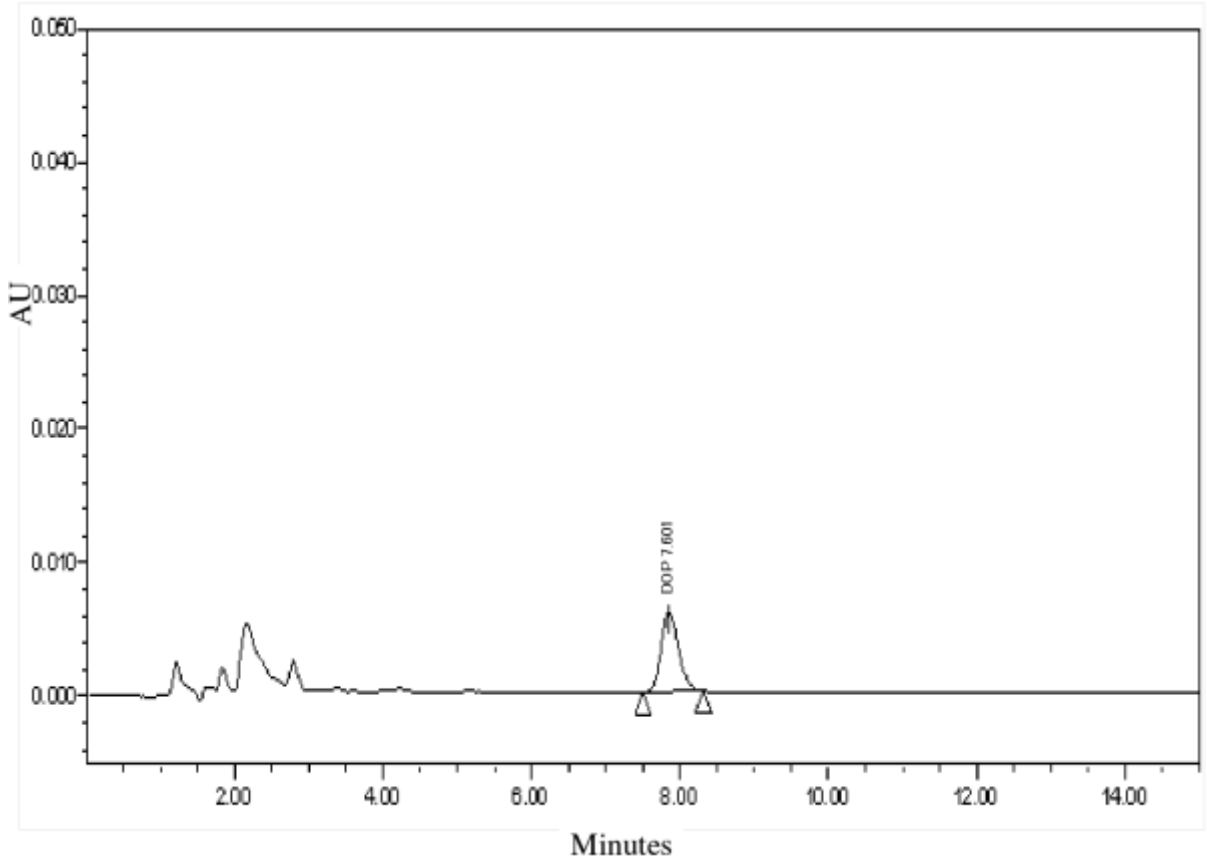
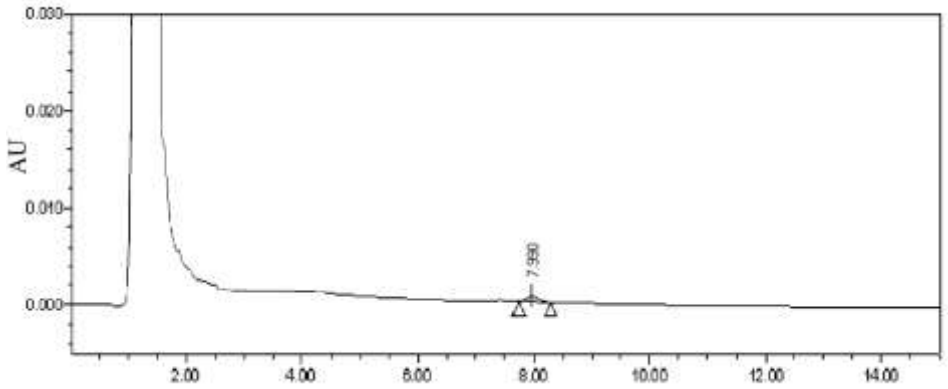


Figure 2. Chromatogram of DOP peak in standard solution.

Peak table

	Name	Retention time, min	Area μV^X sec	USP plate count	USP tailing
1	DOP	7.601	96433	6280	2.38



Pick Table

	Name	Retention time, min	Area μV^X sec	% Area
1	DOP	7.990	9906	100.00

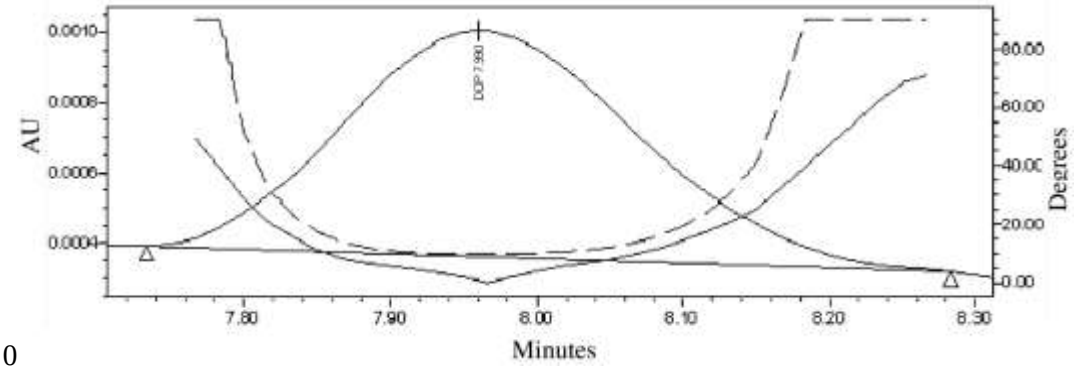


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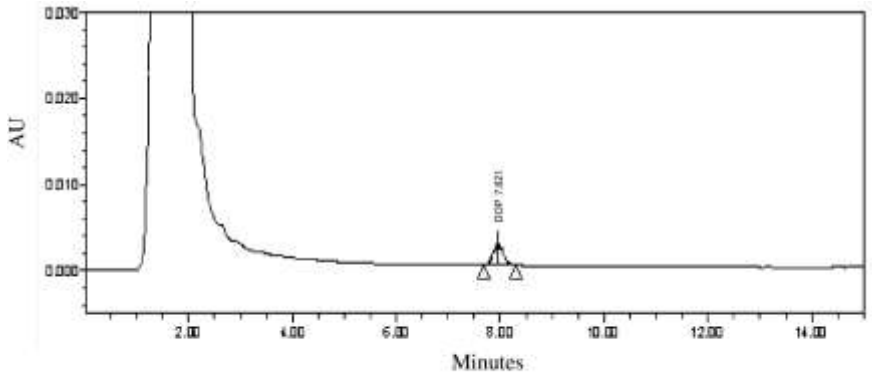
Figure 3. Chromatogram of spiked sample solution in 0.9% Normal Saline Inj. with peak purity plot.



Sr.No.	Name	Retention time, min	Area μV^X sec	% Area
1	DOP	7.821	20664	100.00

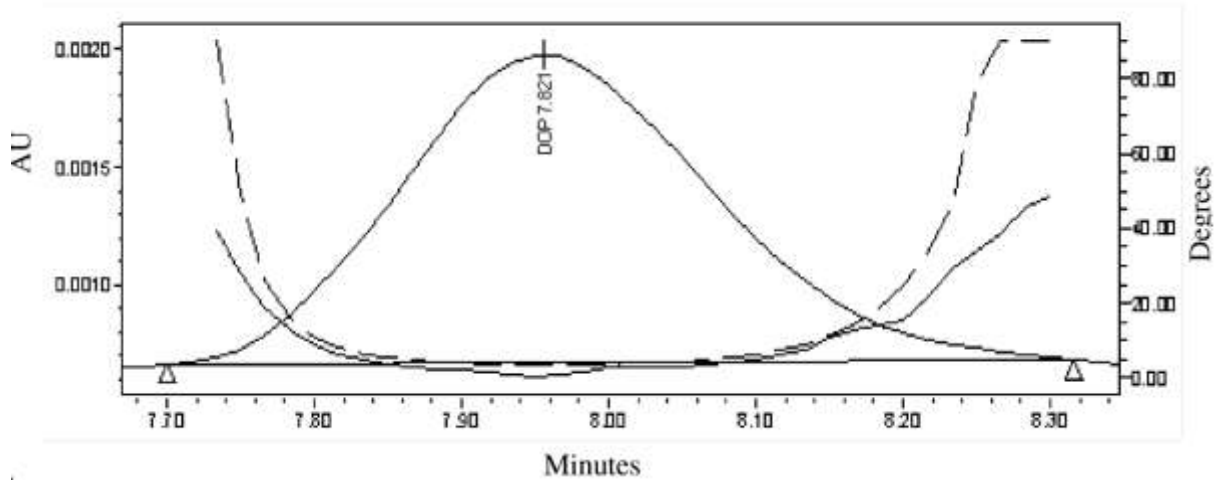


Figure 4. Chromatogram of spiked sample solution in 5% dextrose with peak purity plot.

The system suitability parameters on different days were well met as evident from Table 1. The method was found linear in the concentration range of 0.1 to 8.8 µg $mL^{-1}$of DOP, with a correlation coefficient'R^2'of 0.9995 (Table 2). The limit of quantification for the analytical method was found to be 0.044 µg mL^{-1} (Table 3). A % relative standard deviation of 1.19% for the area counts of DOP peak for six replicate injections of standard solution indicate the system precision (Table 4). Method was found to be precise as indicated by the % relative standard deviation of DOP found 5.28 & 5.17 % with respect to Inj. Diluents (NS 0.9% & DS 5%) content in six sample preparations (Table 5). The percentage recoveries were observed to be in the range of 92.47 to 98.95 % (Table 6). DOP was found to be stable for about 15 hr at RT in 0.9 % Normal Saline Injection Diluent and 5% Dextrose Injection Diluent.

Table 1. System suitability data on different days.

Sr. No.	Column efficiency for DOP peak (No. of theoretical plates) in standard solution	USP tailing for DOP peak in standard solution	% RSD for five replicate injections of standard solution
1	7021	2.23	2.19
2	8730	2.12	1.37
3	8523	2.13	1.52
4	6224	2.16	1.54
5	5397	2.26	2.05
6	6170	2.19	1.65
7	8784	2.14	1.52

Table 2. Linearity of response of DOP.

Conc., µg mL^{-1}	**Mean area counts, µV. sec**
0.150	17268
0.220	31072
0.370	39386
0.740	75636
1.100	99844
1.470	132356
2.850	189960
2.210	260336
3.680	312970
4.420	375798
5.890	505866
7.360	613300
8.830	740856
Slope	**82751**
Intercept	**11040.8**
Correlation coefficient	**0.9995**
LOD	**0.34 µg/ml**
LOQ	**1.03 µg/ml**

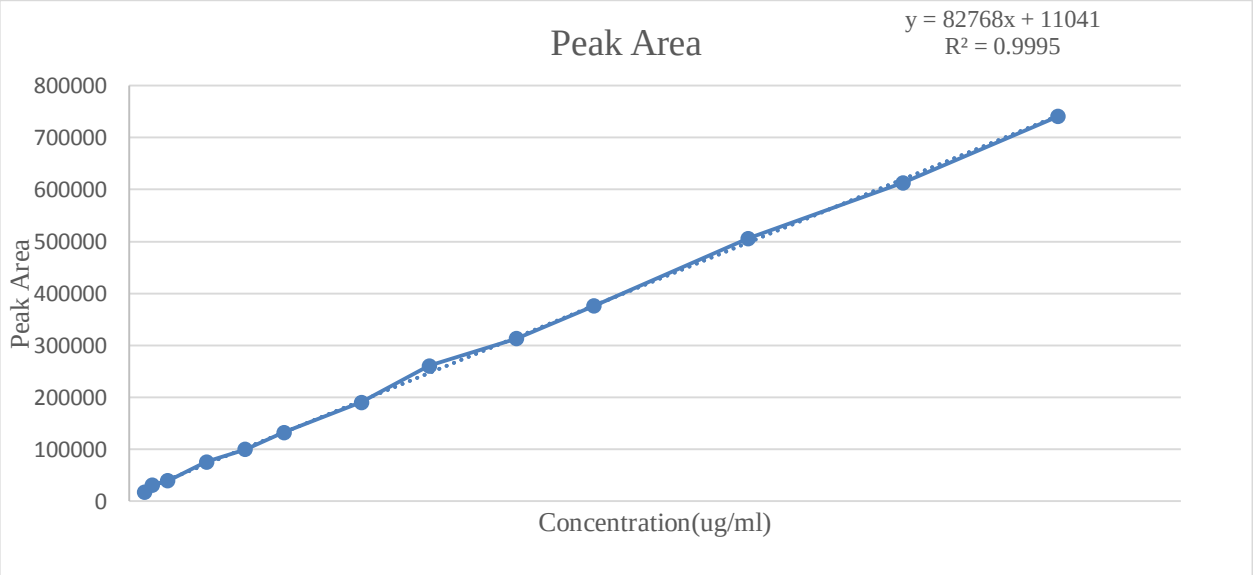


Table 3. Limit of quantification of DOP.

Concentration, µg mL^{-1} **Injection number**	**0.440** **Area counts, µV. sec**
1	17448
2	18154
3	16564
4	17746
5	15260
6	14628
Mean	**16633.33**
SD*	**1423.11**
RSD, % **	**8.56**

* Standard deviation, ** Relative standard deviation

Table 4. System precision.

Injection	Area counts, µV. sec
1	177830
2	174542
3	172004
4	174896
5	173336
6	172688
Mean	**174216**
SD	**2080**
RSD, %	**1.19**

Table 5. Method precision in 0.9% NormaL Saline(NS) and 5% Dextrose Saline Injection.

Sample number	DOP content, µg mL^{-1} (in 0.9% NS)	DOP content, µg mL^{-1} (in 5% DS)
1	0.398	1.592
2	0.366	1.464
3	0.400	1.685
4	0.414	1.656
5	0.362	1.448
6	0.384	1.536
Mean	**0.3873**	**1.563**
SD	**0.0204**	**0.089**
RSD, %	**5.28**	**5.70**

Table 6. Recovery of DOP In 0.9% NormaL Saline(NS) IV and 5% Dextrose Injection

Recovery Level	Amount added, µg	Amount recovered, µg(in 0.9% NS)	% Recovery	Amount recovered, µg(in 5% DS)	% Recovery
Sector-1, Sample Set 1	1.500	1.366	91.07	1.365	92.47
Sector-1, Sample Set 2	1.500	1.402	93.47	1.378	92.47
Sector-1, Sample Set 3	1.500	1.386	92.40	1.421	94.73
Sector-2, Sample Set 1	36.000	34.49	95.81	33.56	93.22
Sector-2, Sample Set 2	36.000	35.29	98.03	34.07	94.64
Sector-2, Sample Set 3	36.000	35.43	98.42	34.89	96.92
Sector-3, Sample Set 1	43.000	42.27	98.30	42.55	98.95
Sector-3, Sample Set 2	43.000	42.68	99.26	42.48	98.79
Sector-3, Sample Set 3	43.000	42.61	99.09	42.80	97.21
Mean			**96.20**		**95.48**
SD			**3.137**		**2.563**
RSD, %			**3.26**		**2.68**

Table 7. Stability of Diluents in Analytical Solution (SIAS).

Time, Hr: Min	SIAS of DOP in standard solution		SIAS of DOP in 0.9% NS Inj.		SIAS of DOP in 5% DS	
	Area	Cum#. %	Area	Cum. %	Area	Cum#. %
0	132293	-	85196	-	10533	-
2:13	132445	0.08	85268	0.06	10711	1.18
4:19	132906	0.22	85599	0.21	10678	0.89
6:30	132765	0.18	86012	0.38	10794	1.02
8:43	132854	0.20	85990	0.35	10829	1.08
10:56	133168	0.25	85726	0.26	10918	1.25
13:09	131372	0.47	85424	0.14	10723	1.14
15:18	133371	0.29	85531	0.36	10862	1.13

Cumulative % relative standard deviation

The maximum DOP concentration in reconstituted injections of CPFX were observed to be 0.13 µg mL^{-1} for sample prepared and stored in 0.9 % Normal Saline Injection Diluent and 5% Dextrose Injection Diluent for 24 hr storage. Both the values were below the tolerance limits (0.3 µg mL^{-1}) (Table 10). There was no increase in content of DOP upon storing the injected IV concentrate for prescribed hours, thereby indicating that DOP did not leach significantly into the reconstituted solution from PVC.

Table 10. Time Analysis of Diluent with Ciprofloxacin Concentrate for injection.

Diluents	Time at Room Temp.	Concentration of DOP µg mL^{-1}
0.9% Normal Saline Inj.	0hr	0.22*
5% Dextrose Inj.	0hr	0.125*
0.9% Normal Saline Inj.	4hr	0.034*
5% Dextrose Inj.	4hr	0.137*
0.9% Normal Saline Inj.	24hr	0.142
5% Dextrose Inj.	24hr	0.136

*Below LOQ

IV. Conclusion

The proposed method is a simple, convenient and sensitive for direct determination of DOP in re-constituting diluents and Ciprofloxacin IV Concentrate for injection. Using this method, analysis time is very short wherein run time is only 15 minutes. Since the same method can be used for various IV Concentrate and respective Diluent, the proposed method is versatile with respect to its applicability in analysing DOP content. Since it is required to be controlled within prescribed tolerance limits as per regulatory bodies, this method can be used as a quality control tool by re-constituting diluent manufacturers for monitoring the level of DOP in re-constituting diluents. Further, this method can be extended to monitor DOP content in other injectable formulations which require reconstitution prior to administration.

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