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Validated Reverse Phase HPLC Method for the Determination of DEHP Content in Reconstituting Diluents and in Reconstituted Solutions of Imipenem and Cilastatin for Injection

A. K. CHAUDHARY, S. ANKUSHRAO WASKE,
S. YADAV, T. G. CHANDRASHEKHAR and VANDANA SINGH*

Analytical Department,
Ranbaxy Research Laboratories, Plot No 20, Sector 18,
Udyog Vihar, Gurgaon, Haryana-122001, India.
vandana.singh@ranbaxy.com

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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 (DEHP) in reconstituting diluents and reconstituted solutions of imipenem and cilastatin for injection The method was applied to detect any leaching enhancement in presence of imipenem and cilastatin in reconstituted injections. Imipenem and cilastatin for injection is a widely used broad spectrum beta-lactam antibiotic. As per requirements of various regulatory authorities of different countries, DEHP content needs to be monitored in the reconstituting diluents used to reconstitute injections. The proposed method is a unique method wherein DEHP 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 DEHP in the reconstituting diluents and reconstituted injections. The method utilizes C_{18} column (250 x 4.6 mm, 5µ) and a mixture of methanol, propan-2-ol and water as mobile phase. DEHP 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).

Keywords: HPLC, DEHP, PVC, FDA, Reconstituting diluents, Imipenem and Cilastatin for injection.

Introduction

Di-(2-ethylhexyl) phthalate, more commonly known as DEHP (CAS No. 117-81-7) is an oily, colourless, viscous liquid with chemical formula C_6H_6 $(C_8H_{17}COO)_2$. It is manufactured using phthalic anhydride and 2-ethylhexanol as raw materials. It is most widely used plasticizer in

polyvinyl chloride (PVC) devices due to higher boiling point (385 °C) providing good heat sustainability. It has low molecular weight (390.56) giving good lubricany and matrix mobility which in turn provides excellent plasticity and softness to PVC[1]. Moreover, PVC being cheap and having excellent varied properties is one among the most commonly used plastic materials for medical devices. DEHP is prominently used in manufacturing PVC medical devices like infusion bags, blood bags, infusion tubings, feeding tubes, catheters *etc*. Virtually all PVC medical devices utilize DEHP as plasticizer. It can leach out from PVC matrix resulting in its exposure to body tissues and fluids[1]. Hence, there are high possibilities of DEHP exposure during medical procedures such as haemodialysis, blood and blood products transfusion, extracorporeal membrane oxygenation, heart bypass surgery and administration of intravenous fluids. The highest human exposures to DEHP can occur in newborns and infants undergoing extensive medical procedures which may lead to a body DEHP level between 130-6000 µg kg body weight^{-1} day[1,2].

DEHP is rapidly metabolized in human body by the enzymes present mainly in the intestines that break down the DEHP into mono-(2-ethylhexyl) phthalate (MEHP). This enzymatic conversion also occurs in the liver, kidney, lungs, pancreas, and plasma. Metabolism of DEHP involves a complex series of reactions that may produce 30 or even more metabolites, prominently MEHP, 2-ethylhexanol, 2-ethylhexanoic acid and several keto acid derivatives that are responsible for its toxicity[3-7]. DEHP also accumulates in adipose tissues[3]. Its significant amount is secreted through milk in lactating females that adversely affects the infants and new born[2].

Studies with laboratory rodents show that DEHP and its metabolites adversely effects reproduction and development produce testicular toxicity, underdeveloped reproductive tracts in males, prominently in infants and new borns[1,2,4]. DEHP exposure may also lead to contact utricaria syndrome, bronco-contraction, immunosupression, mutagenecity and carcinogenicity. Liver, kidney and testes are the other main organs targeted by DEHP induced toxicity[1].

Thus, DEHP leached from PVC medical devices contributing to body fluids and systems is strictly monitored by regulatory agencies. USFDA provides different tolerance limits for adults and neonates of leached DEHP from various PVC medical devices. For intravenous infusion of drugs requiring pharmaceutical vehicles for solubulization, the permissible daily exposure of DEHP is limited to 0.04 mg kg body weight^{-1} day^{-1} for adults (70 kg) and 0.03 mg kg body weight^{-1} day^{-1} for neonates (4 kg)[8].

The diluents used for reconstituting imipenem and cilastatin for injection are 10% dextrose, 10% mannitol, 5% mannitol, 0.9% sodium chloride, 5% dextrose with 0.9% sodium chloride, 5% dextrose with 0.225% sodium chloride and 5% dextrose with 0.02% sodium bicarbonate injection[9.] These reconstituting diluents are supplied in PVC bags, which may lead to DEHP leaching in the reconstituents. Further, presence of imipenem and cilastatin may also contribute to DEHP leaching to reconstituents when stored in PVC bags. As per the pack insert of imipenem and cilastatin for injection, the reconstituted injection may be stored for three to four hours at room temperature (up to 25 °C) or for 24 h under refrigeration (below 4 °C)[9]. Hence, the concentration of DEHP in reconstituents and reconstituted injections both at the initial stage and at extreme storage time points need to be monitored by an appropriate analytical method.

A variety of analytical methods are available for determination of DEHP in various biological and non biological matrixes. Some analytical methods involve pre-concentration

by liquid- liquid extraction of DEHP 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 DEHP in some reconstituting diluents (*e.g.* mannitol) due to precipitation of osmogens[10-11]. Other methods using hexane and ethyl acetate as mobile phase components to monitor DEHP 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 GC[13-15] or involve micro-organic ion association phase extraction[16] to determine DEHP 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 DEHP in reconstituted imipenem and cilastatin injection in various reconstituting diluents[9]. As per dosage regime of imipenem and cilastatin for injection, the maximal acceptable amount of DEHP in reconstituted injection is calculated to be 3.5 µg mL^{-1} for adults and 0.3 µg mL^{-1} for neonates and infants. The limit of quantification of the proposed analytical method is 0.058 µg mL^{-1} which is low enough to quantify DEHP accurately in reconstituted injections much below the tolerance limits[8].

Experimental

HPLC chromatographs, waters 2695 separations module (Alliance) with waters 2996 photo diode array detector (Milford, MA, USA) and agilent 1100 series with agilent G1322A degasser, agilent G1311A quat pump, agilent G1329A ALS, agilent G1330B ALS therm, agilent G1316A colcom and agilent G1315B DAD (Santa Clara, CA, USA) using empower 2 (database version 6.10.00.00) acquisition software were used in analytical method validation. Analytical balances mettler XP205 (Columbus, OH, USA) and sartorius genius (Goettingen, Germany), microbalance sartorius ME5 (Goettingen, Germany) were used throughout the study for weighing. Millipore milli-Q gradient A10 (Billerica, MA, USA), was used for production of HPLC grade water. Millipore millex nylon filters of 0.45 micrometer porosity (Billerica, MA, USA) were used to filter blank, standard and sample preparations. Low temperature incubator (Model 815) used for storage of reconstituted Imipenem and Cilastatin injections at 4 °C was from Precision Scientific (Chicago, IL, USA).

A mixture of methanol, propan-2-ol and water in a ratio of 250:100:50 respectively was used as mobile phase as well as diluent. The analysis was performed using a chromatograph equipped with zorbax extend C18, 250 mm x 4.6 mm, 5 micrometer. HPLC column was thermostated at 30 °C temperature. Ultraviolet detection was done at 225 nm. The retention time of DEHP peak was obtained about 8 minutes with the mobile phase flow rate of 1.5 mL $minute^{-1}$. An injection volume of 100 µL and run time of 15 minutes was found adequate for good response and excellent separation of DEHP. Sample tray temperature was kept ambient. Diluent was used for blank analysis.

To prepare mobile phase and diluent, HPLC grade methanol and propan-2-ol were procured from merck (Mumbai, India) and HPLC grade water was produced from milli-Q gradient A10 water purification system. DEHP working standard (99.74% pure) was procured from Loba Chemie (Mumbai, India). The reconstituting diluents, 10% dextrose, 10% mannitol, 5% mannitol, 0.9% sodium chloride, 5% dextrose with 0.9% sodium chloride and 5% dextrose with 0.225% sodium chloride injection were procured in PVC bags from Baxter (Deerfield, IL, USA). Sodium bicarbonate was used from merck (Mumbai, India). Imipenem and cilastatin for injection (Cilanem) samples were procured from ranbaxy laboratories limited (Dewas, India). Various impurities of imipenem and cilastatin (Imipenemoic acid 1 and 2, 7-Cystein-2-oxheptanoic acid, Impurity A, Imipenem and Cilastatin adduct 1 and 2, Decarboxylated Cilastatin, Δ^3 –Cilastatin, Impurity B and Impurity C) used during validation studies of the proposed analytical method, were procured from ranbaxy research laboratories (Gurgaon, India).

Preparation of standard solution

About 1 g of DEHP standard was accurately weighed into a 100 mL dried volumetric flask, previously containing about 80 mL of methanol. Solution was sonicated with constant swirling to mix the contents and volume was made up to the mark with methanol. 1 mL of this solution was transferred into a 100 mL volumetric 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}.

Preparation of sample solution

Injection vial was reconstituted with 100 mL of reconstituting diluent to get a clear reconstituted solution and 5 mL of this was further diluted to 10 mL with diluent. During in-use 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 insert[9]. 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 DEHP. System suitability parameters, USP tailing (not more than 1.5) and USP plate count (not less than 4000) were monitored for DEHP 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, ruggedness and stability in analytical solution as per ICH[17] guidelines.

Specificity

Specificity of the method was checked by injecting the standard solution, sample solution and sample solution spiked with known related impurities of imipenem and cilastatin in recommended reconstituting diluents.

Linearity of response and limit of quantification (LOQ)

The linearity of the method was checked in the concentration range of 0.07 to 4.4 µg mL^{-1} of DEHP.

Precision

Six replicates injections of standard solution were given to establish system precision. To establish method precision, six samples of single batch of imipenem and cilastatin for injection were prepared independently using 10% mannitol as reconstituting diluent and analyzed.

Accuracy

Known amount of sample was spiked in triplicates with known quantities of DEHP at different levels using 10% mannitol and 10% dextrose as reconstituting diluents. The samples were analysed by the proposed method and amount of DEHP recovered was calculated after making corrections for the amount already present.

Ruggedness

To demonstrate the ruggedness of the method, interanalyst study was performed using 10% mannitol as reconstituting diluent. Six samples of same batch were reconstituted and analyzed by two different analysts on two different days using different chromatographic systems.

Stability in analytical solution (SIAS)

Sample solutions prepared using the diluents 10% mannitol, 10% dextrose and 5% dextrose with 0.02% sodium bicarbonate were injected repeatedly at various time intervals up to about 15 h keeping the sample solutions at room temperature during the study.

Monitoring DEHP content in reconstituted imipenem and cilastatin for injections

Imipenem and cilastatin for injections were reconstituted separately in 10% mannitol, 10% dextrose and 5% dextrose with 0.02% sodium bicarbonate and stored in PVC bags for 24 h at 4 °C and for 4 h at 25 °C.

Results and Discussion

Since the DEHP has chromophoric groups, attempts were made using HPLC with ultraviolet detection to develop the analytical method for determination of DEHP content in aqueous samples. Initially, a chromatograph equipped with zorbax extend C18, 250 x 4.6 mm, 5 µm, utilizing acetonitrile and methanol combination as mobile phase was used to determine DEHP in aqueous samples[10]. Peak shape of DEHP 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 DEHP from reconstituted samples. Again, precipitation was observed using this sample preparation technique. Hence, it was concluded that existing mobile phase composition is not suitable for analysing mannitol based reconstituents.

Several combinations of methanol and acetonitrile were tried as mobile phase to get clear sample solution for mannitol based reconstituents. Precipitation was observed in all the trials. When methanol was used as diluent, initially the sample solution was clear but sample got turbid upon keeping it for some time at room temperature. Hence, a slow precipitation of mannitol was observed with methanol.

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 imipenem and cilastatin for injection. The proposed method was optimized and validated as per ICH[18] guidelines. There was no interference of DEHP peak

from imipenem, cilastatin and related impurity peaks. Peak purity plot of DEHP peak indicated the peak was pure and there were no co-eluting peaks Figure 1-6. This indicated the specificity of the developed method.

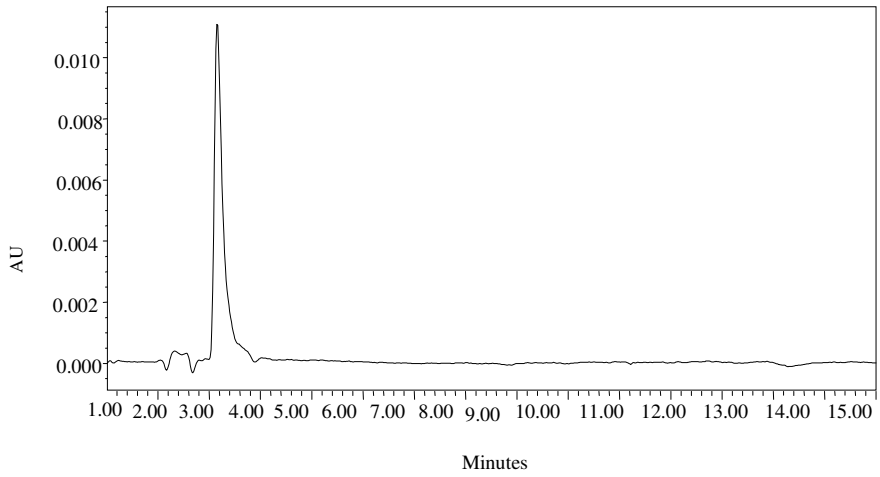
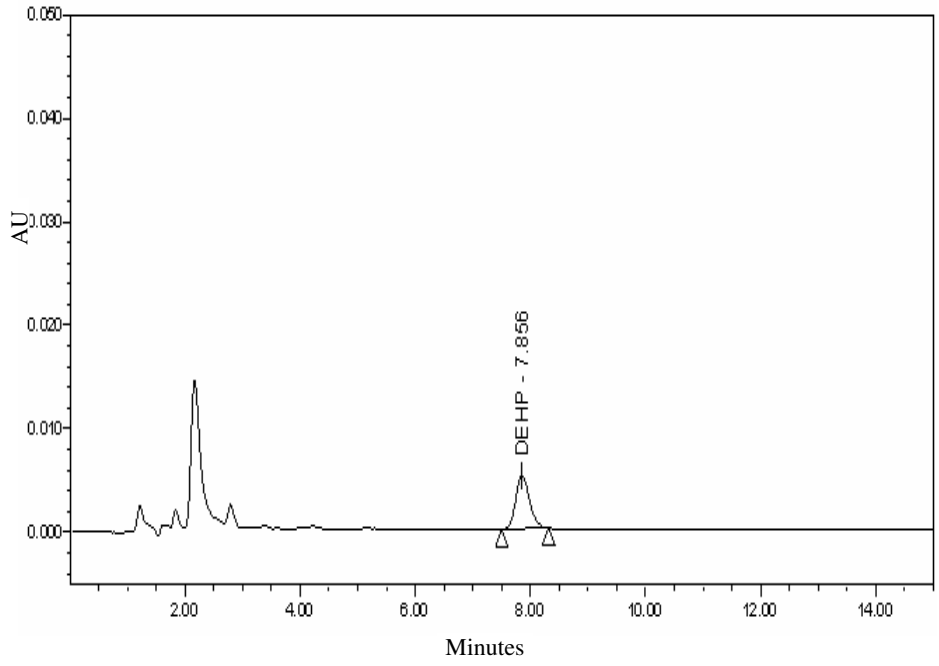


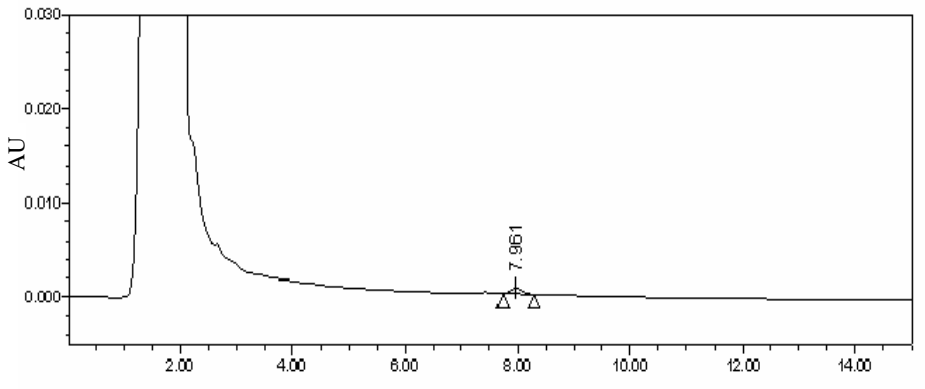
Figure 1. Chromatogram of blank solution.



Peak table

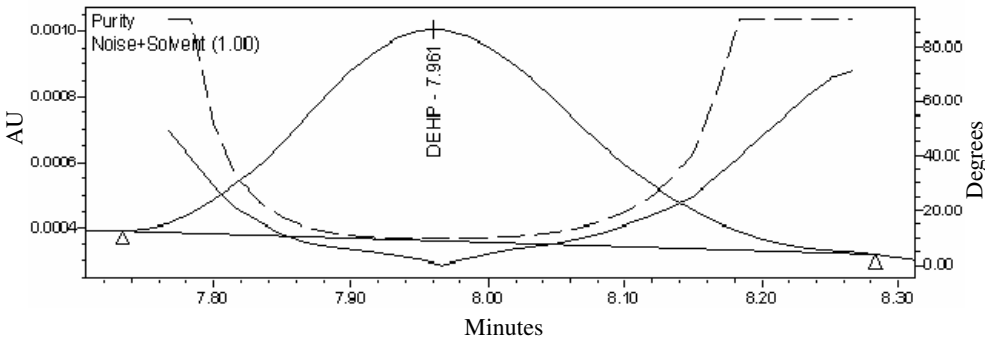
	Name	Retention time, min	Area $\mu V^X sec$	USP plate count	USP tailing
1	DEHP	7.856	85322	5170	1.19

Figure 2. Chromatogram of DEHP peak in standard solution.



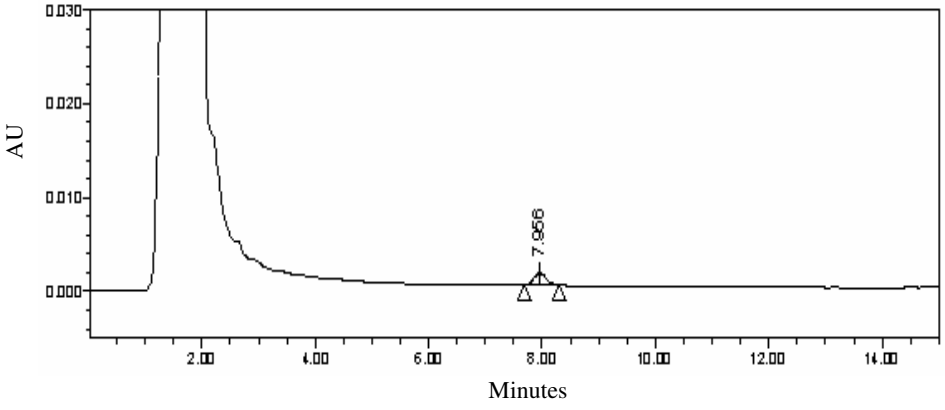
Peak table

	Name	Retention time, min	Area $\mu V^X sec$	%Area
1	DEHP	7.961	8953	100.00



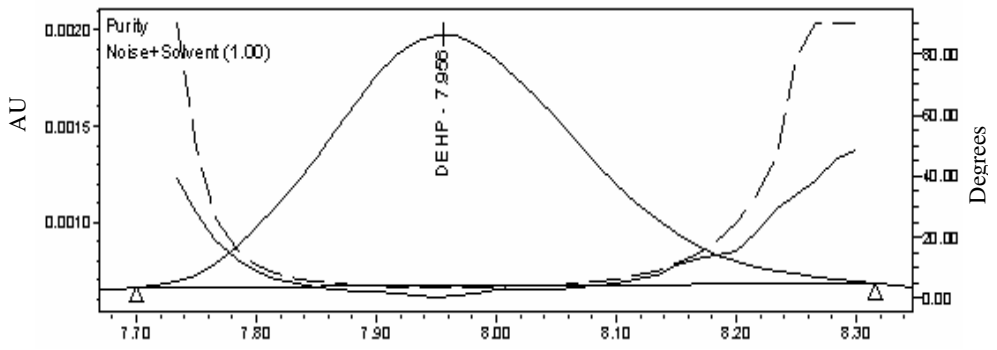
PA: 9.091 TH 13.370

Figure 3. Chromatogram of spiked sample solution in 10% dextrose with peak purity plot.



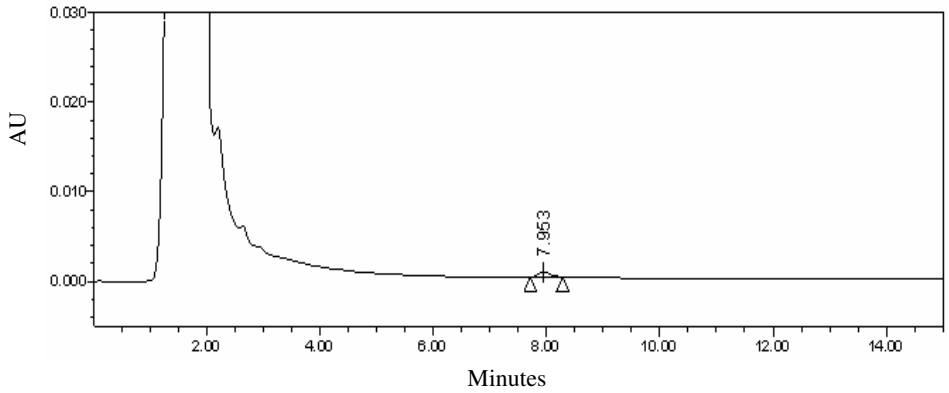
Peak table

	Name	Retention time, min	Area $\mu V^X sec$	%Area
1	DEHP	7.956	19387	100.00



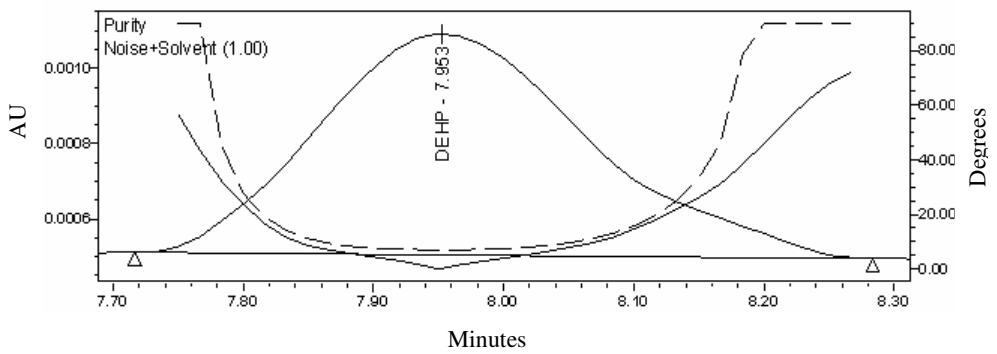
PA: 3.708 TH 4.940

Figure 4. Chromatogram of spiked sample solution in 10% mannitol with peak purity plot.



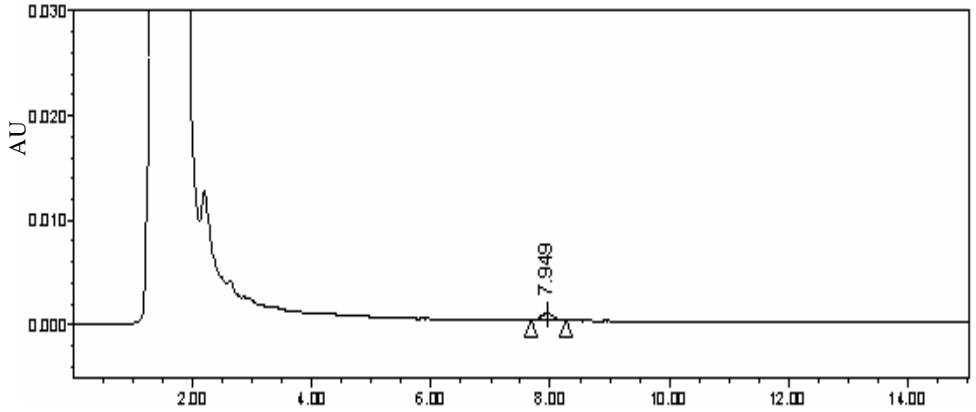
Peak table

	Name	Retention time, min	Area μV^Xsec	%Area
1	DEHP	7.953	8478	100.00



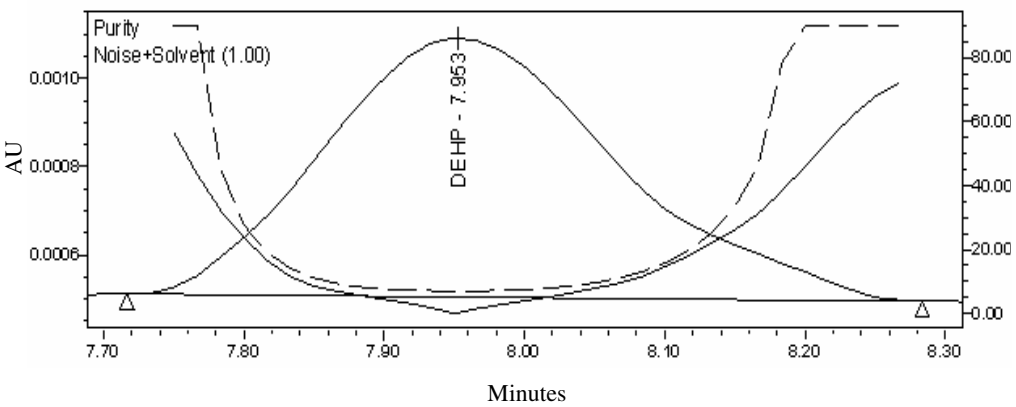
PA: 8.161 TH 9.853

Figure 5. Chromatogram of spiked sample solution in 5% dextrose with 0.02% sodium bicarbonate with peak purity plot.



Peak table

	Name	Retention time, min	Area $\mu V^X sec$	%Area
1	DEHP	7.949	9326	100.00



PA: 10.418 TH 14. 688

Figure 6. Chromatogram of control sample solution in 10% mannitol 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 0.07 to 4.4 µg mL^{-1} of DEHP, with a correlation coefficient of 0.9997 (Table 2). The limit of quantification for the analytical method was found to be 0.058 µg mL^{-1} (Table 3). A % relative standard deviation of 1.19 % for the area counts of DEHP 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 5.26% for DEHP content in six sample preparations (Table 5). The percentage recoveries were observed to be in the range of 93.40 % to 105.67% (Table 6 & Table 7). An overall % relative standard deviation of 6.02% for DEHP content for twelve samples analyzed in two sets demonstrated the ruggedness of the method (Table 8). DEHP was found to be stable for about 15 h at room temperature in 10% mannitol, 10% dextrose and 5% dextrose with 0.02% sodium bicarbonate (Table 9).

Table 1. System suitability data on different days.

S. No.	Column efficiency for DEHP peak (No. of theoretical plates) in standard solution	USP tailing for DEHP peak in standard solution	% RSD for five replicate injections of standard solution
1	6021	1.23	1.19
2	7730	1.12	0.37
3	7523	1.13	0.52
4	5224	1.16	0.54
5	4397	1.26	1.05
6	5170	1.19	0.65
7	7784	1.14	0.52

Table 2. Linearity of response of DEHP.

Conc., µg mL^{-1}	Mean area counts, µV. sec
0.074	8634
0.110	15536
0.184	19693
0.368	37818
0.552	49922
0.736	66178
1.104	95940
1.472	135168
1.840	156485
2.208	187899
2.943	252933
3.679	306650
4.415	370428
Slope	82762
Intercept	5702
Correlation coefficient	0.99969

Table 3. Limit of quantification of DEHP.

Concentration, µg mL^{-1}	0.058
Injection number	Area counts, µV. sec
1	8724
2	9077
3	8282
4	8873
5	7630
6	7314
Mean	8317
SD*	712
RSD, % **	8.56

** Standard deviation, ** Relative standard deviation*

Table 4. System precision.

Injection	Area counts, µV. sec
1	88915
2	87271
3	86002
4	87448
5	86668
6	86344
Mean	87108
SD	1040
RSD, %	1.19

Table 5. Method precision in 10% mannitol.

Sample number	DEHP content, µg mL^{-1}
1	0.199
2	0.183
3	0.200
4	0.207
5	0.181
6	0.192
Mean	0.194
SD	0.0102
RSD, %	5.26

Table 6. Recovery of DEHP In 10% mannitol reconstituted injections.

Recovery Level	Amount added, µg	Amount recovered, µg	% Recovery
Level-1, Sample 1	0.758	0.708	93.40
Level-1, Sample 2	0.758	0.801	105.67
Level-1, Sample 3	0.758	0.771	101.72
Level-2, Sample 1	18.036	17.670	97.97
Level-2, Sample 2	18.036	17.595	97.55
Level-2, Sample 3	18.036	17.668	97.96
Level-3, Sample 1	21.644	21.325	98.53
Level-3, Sample 2	21.644	21.494	99.31
Level-3, Sample 3	21.644	21.805	100.74
Mean			99.21
SD			3.363
RSD, %			3.39

Table 7. Recovery of DEHP in 10% dextrose reconstituted injections.

Recovery Level	Amount added, µg	Amount recovered, µg	% Recovery
Level-1, Sample 1	15.883	15.526	97.75
Level-1, Sample 2	15.883	15.548	97.89
Level-1, Sample 3	15.883	15.522	97.73
Mean			97.79
SD			0.087
RSD, %			0.089

Table 8. Ruggedness.

Sample No.	DEHP content, µg mL^{-1}	
	Set I	Set II
1	0.199	0.213
2	0.183	0.190
3	0.200	0.214
4	0.207	0.184
5	0.181	0.206
6	0.192	0.187
Mean	0.194	0.199
SD	0.0102	0.0136
RSD, %	5.26	6.83
Overall Mean		0.196
Overall SD		0.0118
Overall RSD, %		6.02

Table 9. Stability in analytical solution (SIAS) in reconstituting diluents.

Time, min	SIAS of DEHP in standard solution		SIAS of DEHP in 10% mannitol		SIAS of DEHP in 10% dextrose		SIAS of DEHP in 5% dextrose with 0.02% sodium	
	Area	Cum$^{\#}$. %	Area	Cum$^{\#}$. %	Area	Cum$^{\#}$. %	Area	Cum$^{\#}$.%
0	101553	-	91690	-	91649	-	8877	-
127	101641	0.06	91724	0.03	92156	0.39	8934	0.45
253	99827	1.01	92446	0.46	90792	0.75	8877	0.37
380	101072	0.83	92089	0.39	91780	0.63	9033	0.82
506	101002	0.72	91346	0.46	90746	0.69	8907	0.72
633	99317	0.94	93379	0.79	90921	0.65	8776	0.94
759	100389	0.87	91476	0.77	90130	0.78	8874	0.87
886	100067	0.84	92512	0.73	90968	0.73	8808	0.88

$^{\#}$ *Cumulative % relative standard deviation*

The maximum DEHP concentration in reconstituted injections of imipenem and cilastatin were observed to be 0.15 µg mL^{-1} for sample prepared and stored in 10% mannitol solution for 4 h and in 10% dextrose solution for 24 h storage. Both the values were below the tolerance limits (0.3 µg mL^{-1}) (Table 10). There was no increase in content of DEHP upon storing the reconstituted injections for prescribed hours, thereby indicating that DEHP did not leach significantly into the reconstituting solution from PVC.

Table 10. Analysis in reconstituted solutions of Imipenem and Cilastatin for injection.

Diluents	Time Points	Concentration of DEHP µg mL^{-1}
10% Dextrose	0 H	0.033*
10% Mannitol	0 H	0.135
5% Dextrose+0.02% NaHCO3	0 H	0.037*
10% Dextrose	4 H @ 25 °C	0.044*
10% Mannitol	4 H @ 25 °C	0.147
5% Dextrose+0.02% NaHCO3	4 H @ 25 °C	0.080
10% Dextrose	24 H @ 4 °C	0.152
10% Mannitol	24 H @ 4 °C	0.146
5% Dextrose+0.02% NaHCO3	24 H @ 4 °C	0.058

* *Below LOQ*

Conclusion

The proposed method is a convenient, simple and sensitive for direct determination of DEHP in reconstituting diluents and reconstituted imipenem and cilastain 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 reconstituting dilutents, the proposed method is versatile with respect to its applicability in analysing DEHP 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 reconstituting diluent manufacturers for monitoring the level of DEHP in reconstituting diluents. Further, this method can be extended to monitor DEHP content in other injectable formulations which require reconstitution prior to administration.

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