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# Development and Validation of A Simultaneous Estimation of Darunavir and Cobicistat in Pharmaceutical Dosage Forms Using Micellar Liquid Chromatography

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## ABSTRACT

*A rapid, simple and sensitive liquid chromatographic procedure that use micellar mobile phase is reported for the simultaneous estimation of Darunavir and Cobicistat in pharmaceutical dosage forms has been developed and validated. The separation was performed on an Agilent Polaris C18 (5µm; 150 X 4.6mm) column. The composition of the mobile phase is 10:90 % (v/v) of Propan-2-ol and 0.05mM Tween 80 containing 0.1 % glacial acetic acid in water. Quantification was achieved by HPLC-UV detection at 265 nm. The developed method is validated as per ICH Guidelines over a concentration range from 2.45 – 24.55 µg/mL for Darunavir & 2.52 – 25. 22 µg $mL^{-1}$ for Cobicistat. The method shows excellent linearity and reproducibility. This approach minimizes the usage of organic solvents for analysis.*

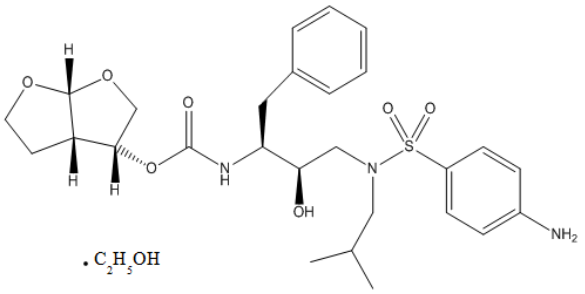
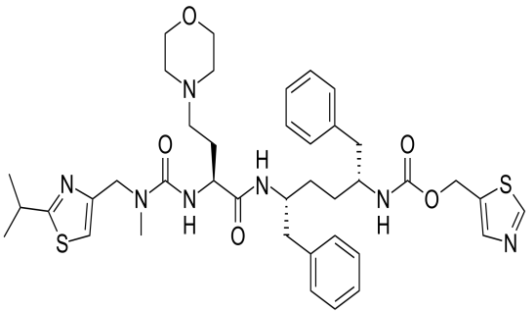
**Keywords:** Cobicistat, Darunavir, Micellar liquid chromatography, ICH.

## INTRODUCTION

Darunavir (Figure 1a), chemically known as " [(3a*S*,4*R*,6a*R*) - 2,3,3a,4,5,6a-hexahydrofuro [2,3-b] furan-4-yl] *N*-[(2*S*,3*R*)- 4 -[(4-aminophenyl)sulfonyl-(2-methylpropyl)amino]-3-hydroxy-1-phenylbutan-2-yl] carbamate", is a antiretroviral drug belonging to the class known as protease inhibitors [1]. Cobicistat (Figure 1b), chemically described as "1,3-thiazol-5-yl methyl *N*-[(2*R*,5*R*)-5-[[(2*S*)-2-[[methyl-[(2-propan-2-yl-1,3-thiazol-4-yl)methyl]carbamoyl]amino]-4-morpholin-4-ylbutanoyl]amino]-1,6-diphenylhexan-2-yl]carbamate", is a pharmacokinetic enhancer and Cytochrome P450 3A Inhibitor [2].

Micellar liquid chromatography (MLC) is a liquid chromatographic technique in which aqueous solutions of surfactant over the critical micellar concentration (CMC) are used as the mobile phase [3-6]. The technique is an interesting alternative because of the lower cost and toxicity, the often improved selectivities, and the separation of compound mixtures of different polarity [7, 8]. The US Food and Drug Administration approved the combination of darunavir and cobicistat for the treatment of human immunodeficiency virus type 1 infection [9-11].

In this paper, we reported the results for the simultaneous quantification of darunavir and Cobicistat using Micellar liquid chromatography (MLC). The method is validated as per ICH Guidelines [12].

| **Figure 1a:** Structure of Darunavir Ethanolate | **Figure 1b:** Structure of Cobicistat |
|---|---|
|  |  |

## MATERIALS AND METHODS

The list chemicals and reagents along with the instrumentation used for the experiment and the optimised chromatographic conditions are given in Table-1 and Table-2 respectively.

**Table-1:** Chemicals, Reagents and Instrumentation

| Chemicals & Reagents | Grade (Manufacturer) |
|---|---|
| Glacial acetic acid | Analytical Reagent Grade (SD Fine Chem Ltd.) |
| Propan-2ol | HPLC grade (Merck ltd) |
| Tween 80 | Best Grade Commercially available (Loba Fine Chem Ltd.) |
| Milli-Q water | In house |
| Darunavir (Purity 99.9 % w/w) | Aurobindo Pharma Ltd. |
| Cobicistat (Purity 99.8 % w/w) | Hetero Drugs Pvt Ltd. |
| HPLC Configuration | Shimadzu Class VP Binary pump LC-10ATvp, SIL-10ADvp Autosampler, CTO-10Avp Column Temperature Oven, SPD-10Avp UV-Visible Detector. SCL-10Avp System Controller was used to control the components of the system. Data analysis was carried out using LC Solutions Version 1.23 software. |

**Table-2:** Optimized Chromatographic Conditions

| Sr. No | Parameter | Description |
|---|---|---|
| 1 | Column | Agilent Polaris C18 (5µm; 150 X 4.6mm i.d) column |
| 2 | Mobile Phase | 10:90 % (v/v) of propan-2-ol & 0.05mM Tween 80 in water containing 0.1% v/v Glacial acetic acid |
| 3 | Flow rate | 1.0 ml / min |
| 4 | Run time | 5 minutes |
| 5 | Column Temperature | 24 ± 1 $^{o}$C |
| 6 | Injection Volume | 20 µl |
| 7 | Detection wavelength | 265 nm |
| 8 | Retention times<br>• Cobicistat<br>• Darunavir | <br>2.13 min<br>3.87 min |

**Preparation of sample solution:** For the estimation in dosage form, 20 tablets [PREZCOBIX] were randomly selected and powdered. Tablet powder equivalent to 1 tablet weight containing 800 mg of Darunavir and 150 mg of Cobicistat was accurately weighed and taken in an extraction flask; followed by 100 mL of diluent solution. This mixture was subjected to vigorous shaking for 30 min for complete extraction of the drug and then centrifuged at 10,000 rpm for 10 min (Biofuge Stratos Heraeus, Kendro

Laboratory Products, and Germany) to remove the excipients that were not soluble in the medium. The supernatant is filtered through 0.45 µm nylon membrane filters and diluted suitably with diluent solution and 20 µL of this solution was injected for HPLC analysis.

**Method Validation:** The method is validated for parameters viz. system suitability, linearity, accuracy and precision (intraday and interday), limit of detection, limit of quantification, specificity from excipients, forced degradation studies and stability (room temperature, auto sampler and long term) studies. Ruggedness and robustness were also carried out as part of validation.

## RESULTS AND DISCUSSION

**Micellar Liquid Chromatography:** MLC shares the basic components of reversed-phase liquid chromatographic (RPLC) systems, that is, a non-polar stationary phase and a polar aqueous mobile phase. The surfactant modifies the nature of the stationary phase and micelles interact with analytes which make the retention mechanism complex. Meanwhile, matrix and other hydrophobic substances are solubilized and can be directly injected into the chromatographic system, which expedites the analytical methodology. Further, they are eluted with, or shortly after, the solvent front, and they do not interfere with analytes [13-16]. In most separations using MLC, hybrid micellar mobile phases in a buffered medium, surfactant monomers, molecules of Organic solvent, and water were used. The organic solvent alters the micelle structure and decreases the polarity of the aqueous solution. For polar compounds short retention times are usually obtained with 1- propanol, 2-propanol and acetonitrile [17]. For non-polar compounds 1-butanol or 1-pentanol are often needed [18]. In the present study Propan-2-ol was used at 10% v/v level as the organic solvent in the mobile phase.

Darunavir being a weakly acidic drug stays unionized at lower pH values. This unionized form of Darunavir usually has no significant interaction with the monomer saturated stationary phase. This lowered silanophilic interactions of the drug with the stationary phase usually causes the elution of the drug at low retention values. On the other hand, Cobicistat is a relatively neutral molecule. Therefore the usage of a non-ionic surfactant is ideal for this combination. The authors employed non-ionic Tween 80 in present experiment at a concentration of 0.05M.

**Modification of stationary phase:** The routine column conditioning for the MLC approach usually involved saturation of the stationary phase with the monomers of the surfactant solution for at least 20 column volumes. In this phase, the surfactant molecules are expected to form a monomer layer across the surface of the stationary phase through electrostatic and hydrophobic interactions. The formation of the monomer layer selectively compartmentalizes and organizes the solute molecules at the molecular level. Solutes are separated based on the differential partitioning between the bulk solvent and micelles in the mobile phase or monomer coated stationary phase. Darunavir being water insoluble, partitioning can occur via direct transfer of solutes between the micellar pseudo phase and the modified stationary phase. Because of the high solubility of Cobicistat in water, the drug remains mostly soluble in its unionized form in the aqueous phase containing the surfactant. This is supported by the unaltered elution behaviour with pH of the mobile phase.

**Solute-Micelle and Solute-stationary phase interactions:** Three partition coefficients viz. (1) PWS: Partition between aqueous solvent and stationary phase (2) PMS: partition between the micelles and stationary phase and (3) PWM: partition between aqueous solvent and micelles operate in the MLC process. PWS and PWM account for solute affinity to the stationary phase and micelles respectively and have opposite effect on retention. With increasing PWS, the retention increases, whereas as an increase of PWM reduces the retention due to the stronger association of the micelles. Neutral solutes eluted with non-ionic and ionic surfactants will be affected by non-polar, dipole-dipole and proton donor-acceptor interactions only[19]. Cobicistat is expected to undergo this process during the elution. While stearic

effects are also important, charged solutes usually undergo hydrophobic and electrostatic interactions with ionic surfactants. Darunavir is expected to undergo this process during the elution. This can be supported by the fact that Cobicistat elutes early and Darunavir later. An RP-HPLC analytical method developed in our laboratory for the simultaneous estimation of Darunavir and Cobicistat found that Darunavir to elute earlier and Cobicistat later under similar stationary phase conditions [Fig-2]. This is perhaps due to the minimal interactions of Darunavir with the stationary phase under high organic solvent strength. Fig. 2 illustrates the chromatograms of the standard solution containing Darunavir and Cobicistat using routine RP-HPLC method.

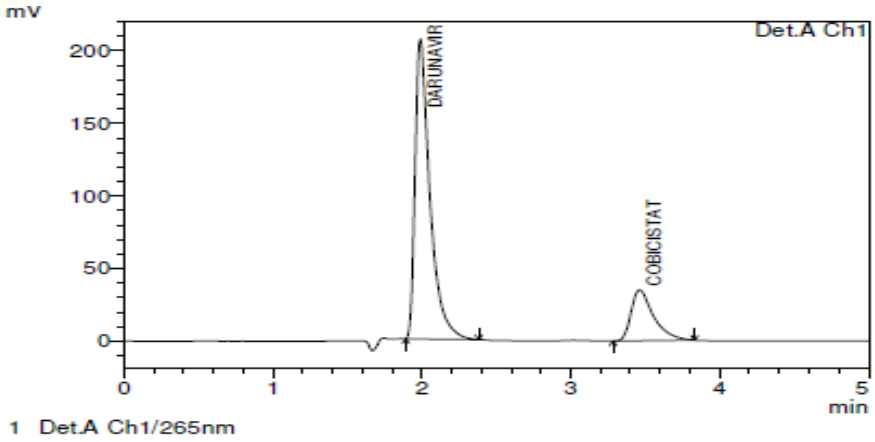



**Fig-2** Chromatogram showing the separation of Darunavir and Cobicistat by RP-HPLC

**Method Validation**

**System Suitability:** The %CV of peak area and retention time for both drugs is within 2% indicating the suitability of the system. The efficiency of the column as expressed by theoretical plates for the six replicate injections and the USP tailing factor is given in Table 3.

**Linearity:** The calibration curve constructed was evaluated by its correlation coefficient. The graph of concentration vs peak area for both the drugs was linear. The Standard deviation of the slopes and intercepts of the calibration curves generated on six different days. The correlation coefficients ($r^2$) of all the calibration curves were consistently greater than 0.999 for both the drugs (Table 3).

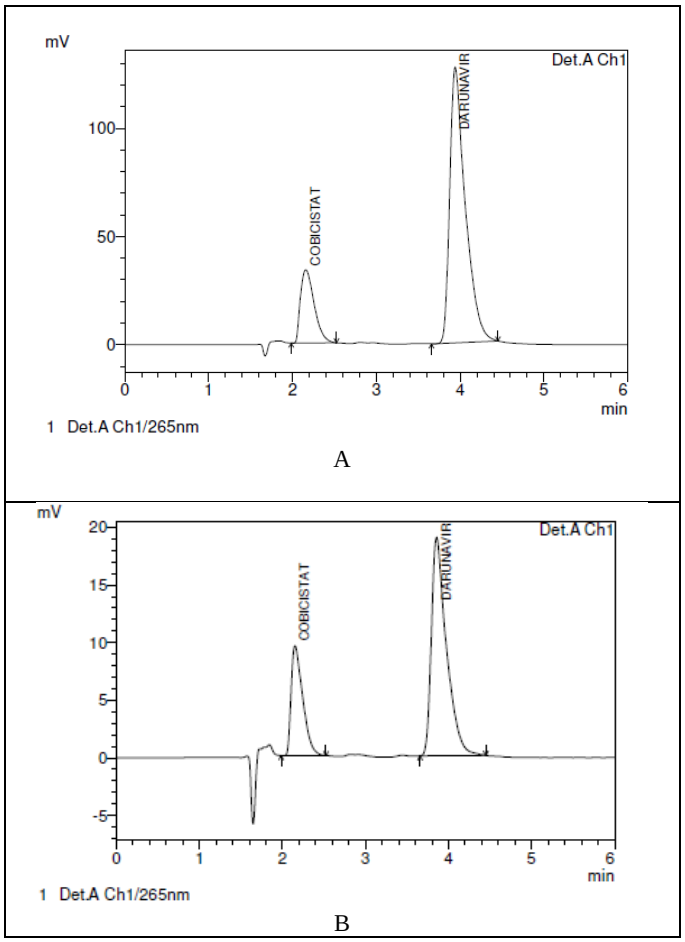
**Precision and Accuracy, Detection and quantification limits (sensitivity):** Fig. 3 illustrates the chromatograms of the standard solution, LOQ of the drug, mobile phase (diluent) and the drug extracted from the formulation. The Accuracy method was found to be good as determined from the six replicate injections (Table 3).

**Table: 3** Results of Validation

| Validation Parameter | Darunavir | Cobicistat |
|---|---|---|
| System Suitability | At sample Concentration of 12.27 µg $mL^{-1}$<br>% CV for Peak Area = 0.39<br>Theoretical Plates: 10815.33±292.28<br>Tailing Factor: 1.63±0.01 | At sample Concentration 12.61 µg $mL^{-1}$<br>% CV for Peak Area = 1.26<br>Theoretical Plates: 3844.17±80.58<br>Tailing Factor: 1.43±0.02 |
| Range | 2.52 – 25.22 µg $mL^{-1}$ | 2.45 – 24.55 µg $mL^{-1}$ |
| Linearity<br>a. Slope | 120544.39 ± 1213.45 | 31117.35 ± 418.45 |
| b. Intercept | 100829.56 ± 1246.15 | 20947.35 ± 1213.45 |
| c. Regression Coefficient | 0.9996 ± 0.0002 | 0.9998 ± 0.0003 |
| Intra and Inter-day assay | Between 97.00 – 103.00 % for all three levels of quality control samples | Between 97.00 – 103.00 % for all three levels of quality control samples |

| Stability | | | | |
|---|---|---|---|---|
| a. Short term (8 hrs) | Between 98.00 – 102.00 % for all three levels of quality control samples | | Between 98.00 – 102.00 % for all three levels of quality control samples | |
| b. Autosampler (24 h) | Between 98.00 – 102.00 % for all three levels of quality control samples | | Between 98.00 – 102.00 % for all three levels of quality control samples | |
| c. Long term (30 days) | Above 95.00 % for all three levels of quality control samples | | Above 95.00 % for all three levels of quality control samples | |
| Specificity | Theoretical Plates | Tailing Factor | Theoretical Plates | Tailing Factor |
| Standard sample | 10994 ± 5.66 | 1.66 ± 0.03 | 5959 ± 7.07 | 1.71 ± 0.01 |
| Oxidation sample | 10645 ± 53.03 | 1.67 ± 0.00 | 3351 ± 38.18 | 1.79 ± 0.01 |
| Alkaline Stress# | 1811 ± 34 | 1.69 ± 0.01 | 3349 ± 53.74 | 1.71 ± 0.01 |
| Acid Stress | 10715 ± 57.28 | 1.69 ± 0.00 | 373 ± 41.01 | 1.70 ±0.01 |
| Photolytic Stress | 10639 ± 31.11 | 1.69 ± 0.02 | 3394 ± 48.79 | 1.69 ± 0.01 |

*Each data is a mean value of 6 replicate injections. # Note that peak split occurred for Cobicistat while Darunavir completely degraded under the alkaline stress conditions.



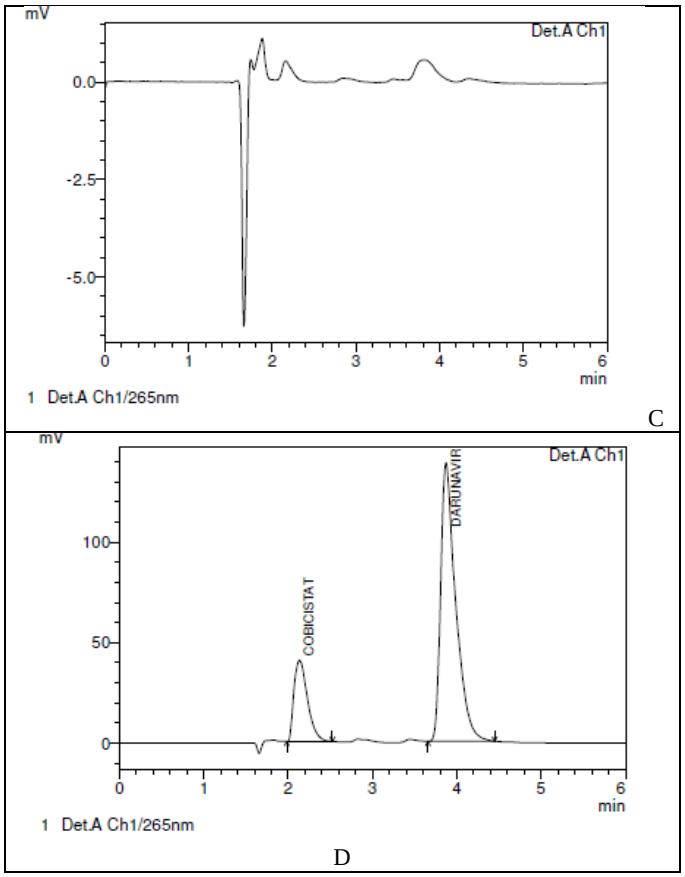
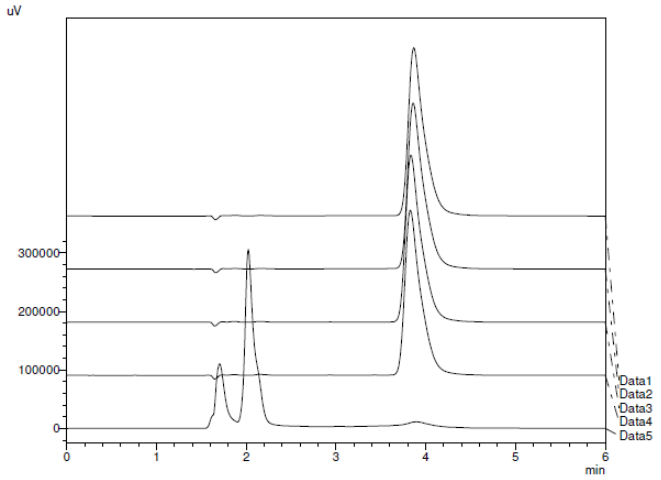


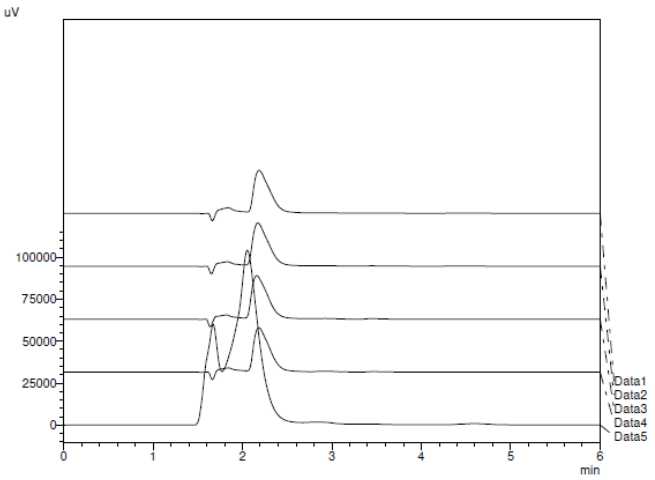

**Figure 3:** Chromatograms of the formulation (a), LOQ (b), Blank (c) and reference solution (d). Darunavir elutes approximately at 3.87 minutes and Cobicistat at 2.13 min.

**Specificity and Stress testing:** Stress testing studies carried out to identify the likely degradation products, the stability of the molecule and also validate the stability and specificity of the analytical procedures (20/ 13). For degradation studies, 100mg of Darunavir and 100 mg of Cobicistat was separately weighed and transferred into separate 50mL volumetric flasks (step 1). To this, 1mL of 1N NaOH (for alkaline degradation) or 1N HCl (for acid degradation) was added and placed in a water bath maintained at 60 ◦C for 1 h. The mixture was cooled, dissolved and made to volume with mobile phase. An aliquot of 1ml of this solution was transferred to a 10-mL volumetric flask, and the volume made up with mobile phase. For oxidative degradation, 1ml of hydrogen peroxide [20] solution was added in step 1. For photolytic degradation, the solution prepared as in step 1 was exposed to UV light at 254 nm for eight hours. After UV treatment, the solution is neutralized, filtered and suitably diluted for analysis. The final solution was injected for analysis. From the fig. 4a and 4b, the degradation products are clearly separated. The specificity of the analytical method was indicated in fig. 3. Specificity data indicated alkaline instability for Darunavir and Cobicistat (Fig 4a and Fig 4b).




Data 1 – Fresh Sample; Data 2 - Acid Stress; Data 3 - Oxidative Stress; Data 4 - Photolytic Stress and Data 5 – Alkaline Stress

**Figure 4a:** Chromatograms depicting the specificity of the method for Darunavir by MLC




Data 1 – Fresh Sample; Data 2 - Acid Stress; Data 3 - Oxidative Stress; Data 4 - Photolytic Stress and Data 5 – Alkaline Stress

**Figure 4b:** Chromatograms depicting the specificity of the method for Cobicistat by MLC

**Stress testing:** The stress studies involving heat and acid, alkaline stress, light (UV), oxidation and reduction revealed that Darunavir and Cobicistat were not fully degraded (Table 4). In separate experiments that were performed in our lab, we tested Darunavir and Cobicistat using RP-HPLC methods in which the diluent solution was a solution of 50% methanol in water. We noticed that the column and wavelength were same as that used for the present MLC approach. In both the RP-HPLC method and MLC approach, we noticed a sharp decrease in the stability of Darunavir under alkaline conditions. In the RP-HPLC method for Darunavir, the degradation peak of Darunavir due to alkaline stress eluted early perhaps due to the reduced molecular size of the degradation product accompanied with a drastic reduction in the number of theoretical plates and increased tailing (Table 3).

## APPLICATIONS

The HPLC method developed using the MLC approach is sensitive and specific for the quantitative determination of Darunavir and Cobicistat in pharmaceutical dosage forms. Also, the method is validated

for different parameters. PREZCOBIX tablets containing 800 mg Darunavir and 150mg of Cobicistat strength were evaluated for the amount of Darunavir and Cobicistat present in the formulation. Each sample was analysed in triplicate after extracting the drug as mentioned in the sample preparation of the experimental section. The amount of Darunavir in both the formulations was between 98.65% and 99.85% while that of Cobicistat was between 99.76 – 100.12 %. None of the tablet ingredients interfered with the analyte peak as seen in fig. 3.

## CONCLUSIONS

A simple Micellar liquid chromatographic method for the simultaneous estimation of Cobicistat and Darunavir in pharmaceutical dosage forms was described. ICH guidelines followed to validate the reported method. The MLC approach will play a dominant role in replacing RP-HPLC methods thereby reducing the toxic disposals generated during routine laboratory experiments.

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