

A Simple and Efficient Approach for Estimating Recovery of a Preparative Reversed Phase HPLC Purification

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Abstract The widely applied reversed phase high-performance liquid chromatography (RP-HPLC) is an indispensable purification technique in drug discovery. During drug discovery, recovery was usually calculated based on the weight of the purified product after drying over the weight of the crude material multiplied by the assumed purity from HPLC/UV area percent of the product. Such a purity assumption can be off significantly when the crude material contains water, solvents, other UV-inactive impurities and inorganic salts. In this paper, we report a simple and efficient way to estimate recovery of preparative HPLC purification process. It is based on the ratio of the HPLC/UV peak area measured for the product in the crude solution and that in the final collected fraction with both accounted for their volumes. This approach eliminates not only the need for drying of the collected fraction to calculate recovery but also the inaccuracy associated with the true content in the crude sample using the traditional method. A systematic study was conducted to verify this method using caffeine mixed with various UV-active and -inactive impurities. The calculated recoveries using this approach were found to be consistent within 4% with the true recoveries based on dry weight estimation. The approach has been successfully applied for our in-house purifications. Furthermore, the approach was extended to library purifications,

where in many cases heart-cutting the desired peaks is used to meet the purity requirements.

Keywords Preparative purification · Sample recovery · Reversed phase HPLC · Caffeine

Introduction

Reversed phase high-performance liquid chromatography (RP-HPLC) is a preferred purification technique in drug discovery. Although the value of HPLC purification is well acknowledged [1] and high-throughput purification is increasingly reported [2, 3], there is little discussion on fast recovery assessment during the HPLC purification in the literature. A reliable recovery assessment of HPLC purification process is important for developing and optimizing synthesis and purification of often precious target drug molecules. One of the earlier reported methods for recovery measurement makes use of commercially available drugs or drug-like compounds as standards and the estimated recovery is calculated by comparing the weight of the compound of interest before and after purification [4]. Although this can confirm the instrument and the method performance, the question still remains unanswered about the purification of a real sample where many other factors have to be considered. Usually, the weight of the compound of interest before purification is estimated by multiplying the purity of the component with the weight of the crude material. Thus, accurate recovery determination depends on the accurate assessment of the purity of compound of interest in the crude material. During drug discovery, since reference standard of a target drug molecule is rarely available, HPLC/UV peak area % is used for purity assessment of the crude material and final product. When crude material contains UV-inactive

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components such as inorganic salts, moisture and solvents, the purity of the crude could be off significantly. As a result, purification recovery could be underestimated and becomes a point of concern. To the best of our knowledge, there is no reported method for recovery assessment which is independent of the weight and purity of the crude material.

Zhang et al. reported mass spectroscopy (MS) directed automated HPLC purification [4, 5], which enhanced the reliability of fraction collection and automation. Nowadays, preparative HPLC/MS instruments have been widely adopted for purifying library compounds. However, MS can selectively detect the target component even with co eluting peaks, HPLC/MS does not help in purity and recovery determination.

There are a few methods reported for the use of HPLC with a chemiluminescent nitrogen detector (CLND) or ELSD as a means of estimating compound concentration in library purifications [6–10]. These methods were used as quality control check for the assessment of the purity and concentration of compound in the DMSO solutions for high-throughput screening after the purification. Although these methods are viable, these detectors are not as trouble-free as UV detectors especially for crude samples. CLND also has the limitations of using nitrogen-free mobile phase. ELSD can not respond well towards volatile and semi-volatile compounds and has limitation of non-linear response curve with gradient change in the mobile phase composition. Furthermore, none of these detectors offer a true universal response for a wide variety of compounds for purity determination. In order to determine recovery quickly for HPLC preparative purifications, we developed a simple and efficient HPLC/UV approach which is independent of the weight and true purity of the crude or the dry weight of the purified compound. At the same time, this approach will be independent of the presence of impurities with much higher or lower UV response in the crude sample relative to the product. The approach involves the HPLC injections of crude sample solution as well as the collected fraction after the purification. By comparing the peak area of the compound of interest accounted for the volumes of the crude sample solution and post-preparative HPLC fraction, one can calculate the amount in the crude sample versus the amount in the fraction solution which reflects the recovery of the HPLC purification process. Of course, the approach does not cover any loss or decomposition that might occur in the final drying. Therefore, characterization of the final dried compound is required especially when loss or decomposition is suspected. Herein, we demonstrate our approach using caffeine mixed with different amount of UV-inactive impurities, impurity with high UV response, water, solvent and inorganic salts. HPLC purification recovery by this method was compared with the true recovery calculated based on dry weights. This process has been implemented

for the routine recovery assessment for all the compounds which are purified in our drug discovery facility.

Materials and Methods

Reagents and Chemicals

HPLC grade acetonitrile was purchased from Rankem (Avantor Performance Materials Ltd, Ahmedabad, India). Water was purified and delivered by Millipore's Milli-Q system (Bedford, MA, USA).

Sodium sulphate was purchased from Spectrochem (Milwaukee, WI, USA). HPLC-grade methylsulfoxide (DMSO), 6-amino benzothiazole and naphthalene were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Caffeine was purchased from Aldrich (St. Louis, MO, USA). In-house compounds used for the recovery study were produced by the chemistry group.

HPLC and MS Instrumentation

The purification system consisted of an Agilent (Santa Clara, CA, USA)-1200 series (German-1361A) preparative HPLC system, which included two solvent delivery pumps (G2260A), one DAD detector (G1315D) and one system controller (G1364D) to control the HPLC gradient. Sample injection and collection were controlled by Chemstation software.

Columns, Mobile Phases and Preparative HPLC Conditions

All preparative HPLC separations were carried out using Sunfire OBD C18, 30 mm × 250 mm, 5 μ (waters) column with analytical method developments on Sunfire OBD C18, 4.6 mm × 250 mm, 5 μ (waters) column. Milli-Q water and acetonitrile were used as mobile phase for all separations and purifications. Diluents for all sample preparations were 80:20 acetonitrile:water (V/V). The gradient used for analysis and preparative purifications are as shown in Table 1.

Table 1 Gradient profile for the analytical and prep loading

Analytical gradient		Preparative gradient	
Time	% B (acetonitrile)	Time	% B
0	0	0	05
8	20	8	20
15	20	12	20
18	100	15	95
22	100	21	100
22.5	00	22	05
26.0	00	24	05

Sample Preparation

Following three compounds were used for the preparation of test mixture 2.

Naphthalene Caffeine 6-Amino benzothiazole

Eleven test mixtures were prepared for the recovery study to mimic the actual sample components which may interfere with recovery assessment in purification:

Mixture 1 97.5 mg of caffeine was dissolved in 1.5 mL of diluent.

Mixture 2 52.5, 53.6 and 52.3 mg of caffeine, 6-amino benzothiazole and naphthalene, respectively, were dissolved in 1.1 mL of diluent.

Mixture 3 98.5 mg of caffeine and 60.2 mg of Na₂SO₄ was dissolved in 4 mL of diluent.

Mixture 4 101.2 mg of caffeine and 50.1 mg of DMSO were dissolved in 1.5 mL of diluent.

Mixture 5 300.5 mg of caffeine was dissolved in 2 mL of ACN and 3 mL of water, and the sample was transferred to a 100 mL round bottom flask and the solvents were evaporated in such a way that the compound did not dry completely. 110.3 mg of this sample was dissolved in 1.2 mL diluent.

Mixture 6 50.2 mg of caffeine and 54.5 mg *N*-*boc*-*trans*-4-hydroxy-*L*-prolin methyl ester (UV inactive) were dissolved in 1.1 mL of diluent.

Mixture 7–11 30.9 mg ammonium chloride, 34.6 mg potassium chloride, 31.0 mg sodium chloride, 31.4 mg sodium bicarbonate, and 38.6 mg calcium chloride were mixed with caffeine separately in approximately 1:0.3 ratios (wt/wt) and dissolved in 1 mL diluents.

Mixture 12 10.2 mg of caffeine and 11.6 mg of an impurity with high UV response were dissolved in 1.1 mL of diluent. All mixture solutions were injected completely on the prep column in 1–5 injections (depending on the solution volume) with an injection volume of 1.0 mL. The peak corresponding to caffeine was collected and evaporated by lypholization to get the final solid which was weighed for actual wt/wt recovery. Crude sample solution and the final fraction (combined fraction from multiple injections) were injected on an analytical HPLC to get the peak area for prep recovery calculation. Crude samples were diluted 'n' folds as appropriate before injection to avoid the UV detector saturation (peak height <800 mAU) in the analysis, and n has been incorporated as a dilution factor in the recovery calculation formulae.

Results and Discussion

We have developed a simple method to assess HPLC purification recovery for each sample without the need to

determine the exact weights of the component of interest before and after the purification. The recovery is calculated based on the peak areas of the component of interest accounting for the volumes in the crude sample solution and in the collected fraction (all fractions were pooled together at the completion of the purification and recovery estimation was done from this pooled solution.) using the following formulae:

$$\% \text{ Sample recovery} = \frac{A_f \cdot V_f \cdot \text{Inj}_c \cdot 100}{A_c \cdot V_c \cdot \text{Inj}_f \cdot n}$$

where A_f is the peak area in the final collected fraction; V_f is the fraction volume; Inj_c is the injection volume of the crude sample; A_c is the peak area in crude; V_c is the crude volume; Inj_f is the injection volume of the fraction; n is the dilution factor of the crude sample solution for HPLC injection.

Based on the equation, the factors contributing to the accuracy of the recovery result include accuracy of the volume measurements (crude and fraction volume), performance of the analytical HPLC used for the analysis and proper dilutions to ensure the concentration of crude and collected samples are within the detector linear range. Properly controlling these experimental factors can result in accurate recovery estimation to the nearest 1%. Usually recovery in the actual sample is affected due to the presence of following components- UV active impurities, inorganic impurities, water, solvent and UV inactive impurities. To demonstrate our approach, we prepared eleven samples of caffeine mixed with various amount of UV-active and UV-inactive components including water, DMSO and salts. The mixtures were subjected to prep-HPLC purification and the caffeine peak was collected. The final collected fractions as well as the crude mixtures (after dilution) were injected on an analytical HPLC. The recovery was calculated for the HPLC purification of each mixture sample using the proposed formulae. The results are listed in Table 2. Representative chromatograms for mixture 2 are shown in Figs. 1, 2, 3. Figure 4 shows the caffeine mixture with an impurity of high UV response. Actual recovery (wt/wt) was also calculated for comparison using the following formulae:

$$\% \text{ wt/wt recovery} = \frac{\text{Weight of caffeine recovered} \times 100}{\text{Weight of caffeine injected}}$$

Data in Table 2 clearly shows that the traditional approach yielded significantly larger error (from 4.65 to 48.98%) in the recovery assessment than the new proposed method (from 0.01 to 3.74%) especially for the purifications of the samples containing UV inactive impurities. This confirms that the proposed HPLC area-based recovery calculation method is not affected by the presence of these UV-active or

Table 2 Calculated and actual recoveries of the mixture samples after the preparative purification

Mixture no.	(A): Other component and quantity in sample (mg)	(B): Amount of caffeine added (mg)	(C): Total weight (Caffeine + additive)	(D): % Purity of caffeine in crude sample by HPLC peak area %	(E): Traditional calculated weight of caffeine in crude sample (mg) (C*D/100)	(F): Amount of caffeine recovered by drying and weighing (mg)	(G): Actual recovery based on amount of caffeine recovered vs. added (wt/wt%) (F/B*100)	(H): Traditional calculated recovery (F/E*100)	% Error in recovery by traditional calculation	(I): Calculated recovery using the new approach	% Error in recovery by new approach
1	None	97.5	97.5	99.91	97.41	92.3	94.67	94.75	-0.09	95.70	-1.09
2	6-Amino benzothiazole (53.6) and naphthalene (52.3)	52.5	158.4	38.14	60.41	49.9	95.05	82.60	13.10	95.80	-0.79
3	Na ₂ SO ₄ (60.2)	98.5	158.7	99.9	158.54	94.3	95.74	59.48	37.87	97.30	-1.63
4	DMSO (50.1)	101.2	151.3	70.15	106.14	91	89.92	85.74	4.65	91.10	-1.31
5	Water (35.34%)	110.3*	110.3	99.85	110.13	67.1	94.08	60.93	35.24	97.60	-3.74
6	UV inactive (54.5)	50.2	104.7	93.98	98.40	46.9	93.43	47.66	48.98	94.10	-0.72
7	Ammonium chloride(NH ₄ Cl)-(30.9)	103.8	134.7	99.97	134.66	103.1	99.33	76.56	22.92	99.20	0.13
8	Potassium chloride(KCl)-(34.6)	103.2	137.8	99.97	137.76	102.6	99.42	74.48	25.09	96.40	3.04
9	Sodium chloride (NaCl)-(31.0)	100.8	131.8	99.88	131.64	99.6	98.81	75.66	23.43	98.80	0.01
10	Sodium bicarbonate (NaHCO ₃)-(31.4)	102	133.4	99.94	133.32	99.3	97.35	74.48	23.49	97.30	0.05
11	Calcium chloride (CaCl ₂)-(38.6)	101.5	140.1	99.97	140.06	100.2	98.72	71.54	27.53	98.70	0.02
12	High UV active compound (11.6)	10.2	21.8	31.65	6.90	9.4	92.16	136.24	-47.83	94.7	-2.76

* Actual amount of caffeine in mixture 5 was 71.32 mg after taking out 35.34% of moisture

Fig. 1 Preparative chromatogram of Mixture 2 containing caffeine, 6-amino benzothiazole and naphthalene. Y axis presents peak area (mAU) and X axis run time (min)

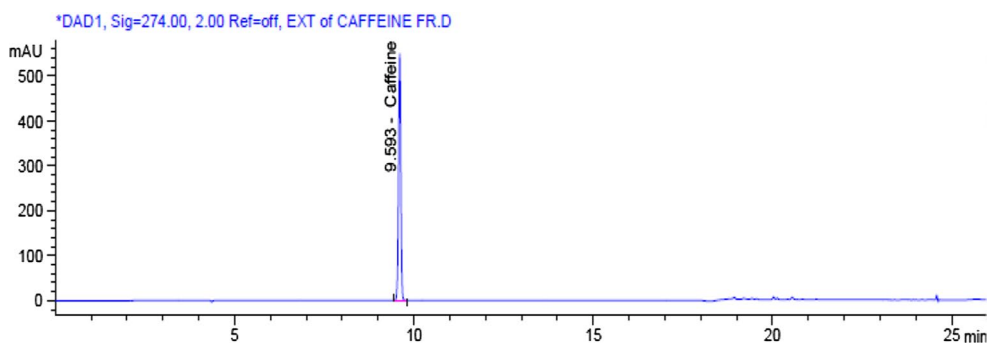
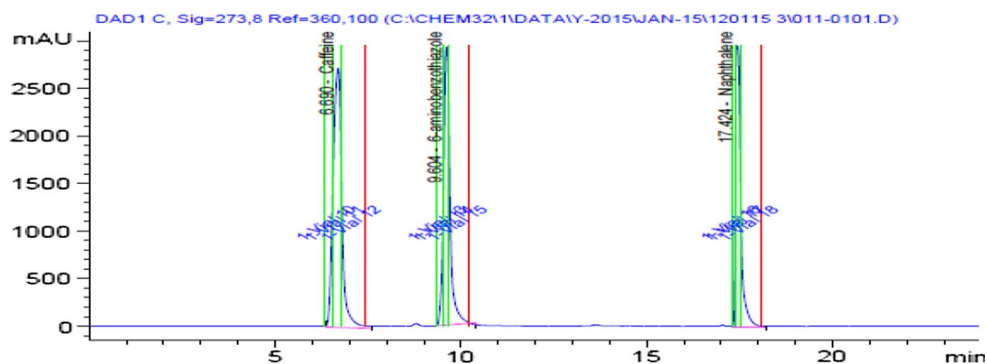
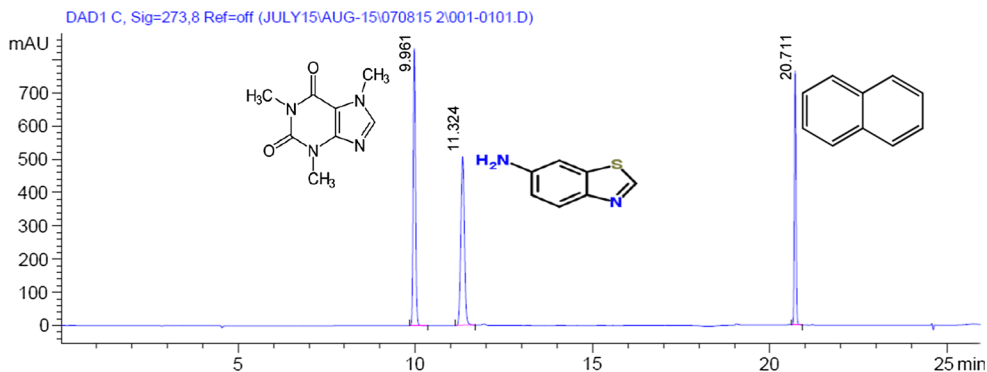


Fig. 2 Analytical chromatogram of fraction 2 (after prep purification) of Mixture 2 containing caffeine. Y axis presents peak area (mAU) and X axis run time (min)

Fig. 3 Analytical chromatogram of diluted Mixture 2 containing caffeine, 6-amino benzothiazole and naphthalene before prep. Y axis presents peak area (mAU) and X axis run time (min)



inactive components in the crude samples. If any UV inactive impurity is co eluting with the peak of interest the present recovery estimation may still give the right recovery as it is solely based on the HPLC peak area but the recovery based on final dried weight of the compound may differ due to addition of this impurity in final weight. That may be verified by further method of development to separate the undesired impurity or with the help of other techniques like NMR or MS to cross check the purity of the sample.

In case of close eluting peaks with difficult separations during the library purifications, it is a normal practice to heart-cut the fractions by removing the peak fronting

or tailing which leads to a lower recovery. To check the applicability of this method for heart-cut fraction purifications, we collected the caffeine peak in three fractions and checked the recovery of each fraction separately using the proposed area-based method and wt/wt method. Figure 5 shows the chromatogram of caffeine with heart-cut fraction collection.

As shown in Table 3, the separate recovery from each fraction as well as overall recovery including all fractions is very close to the dry weight recovery assessments. This also confirms that around 75% material could be recovered if only the middle fraction is taken for high purity

Fig. 4 Analytical chromatogram of Mixture 12 containing caffeine and an impurity of high UV response. Y axis presents peak area (mAU) and X axis run time (min)

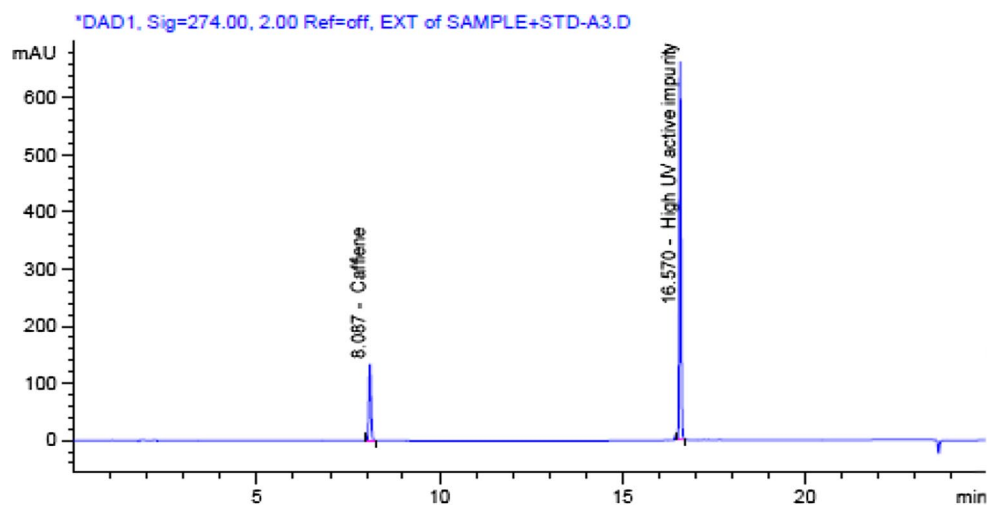


Fig. 5 Prep-HPLC Chromatogram with heart-cutting fraction collection. Y axis presents peak area (mAU) and X axis run time (min)

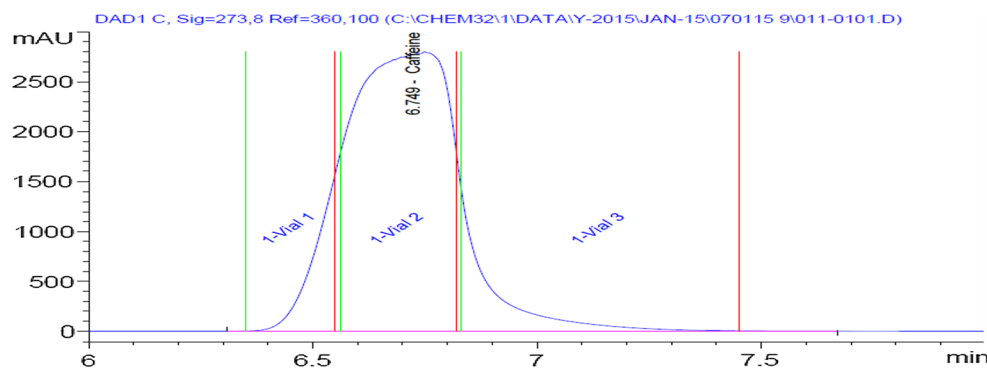


Table 3 Summary of recovery in three fractions from the heart-cut experiment

Sample	Fraction volume (mL)	Peak starting (min)	Peak end (min)	Flow rate (mL)	Number of injections	Caffeine recovery (mg)	% Prep recovery (area/area)	% Actual recovery (wt/wt)
Fr-1	16.2	6.35	6.55	27	3	6.3	6.61	6.46
Fr-2	21.06	6.56	6.82	27	3	71.8	74.87	73.64
Fr-3	50.22	6.83	7.45	27	3	14.2	14.20	14.56
						Total % recovery	95.68	94.67

requirement which could be helpful to make a decision for the collection of fractions.

This area-based approach requires the measurement of the fraction volume for the recovery calculation which would add additional time and efforts for each sample. To further simplify the procedure, the fraction volume could also be calculated directly from the chromatogram using the following formulae:

$$V_f = (R_{t_{\text{end}}} - R_{t_{\text{start}}}) \times \text{flow rate} \times \text{number of injections.}$$

where $R_{t_{\text{end}}}$ is the retention time at the end of peak collection, and $R_{t_{\text{start}}}$ is the retention time at the start of peak collection.

However, recovery calculation using this formula may not be accurate if there is any instrumental error in terms of the retention volume during multiple injections.

Conclusion

We report a simple and efficient approach to assess the recovery of a prep-HPLC purification based on the peak area of the component of interest in the crude and in the collected fraction solutions. This approach does not require the determination of weight and % purity of the crude sample which is often inaccurate especially when the sample contains UV-inactive impurities. The method was demonstrated to be reliable using caffeine mixed with various UV-active and inactive components. This process has been implemented for the routine recovery assessment for all the compounds which are purified in our drug discovery facility. In addition, it also worked well for the assessment of recovery for different fractions of the peak collected so it can be used successfully for library purifications as well.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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