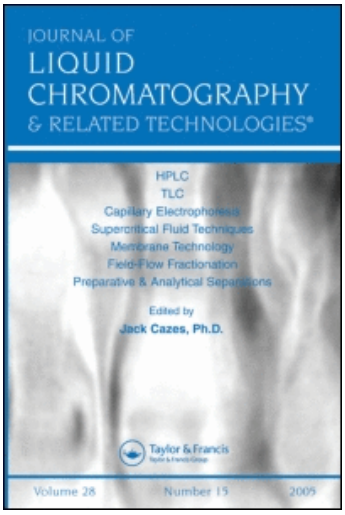


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# Simultaneous Determination of Dexamethasone, Dexamethasone 21-Acetate, and Paclitaxel in a Simulated Biological Matrix by RP-HPLC: Assay Development and Validation

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**Abstract:** A simple and accurate reversed-phase high performance liquid chromatography method was developed and validated for the simultaneous determination of dexamethasone, dexamethasone 21-acetate, and paclitaxel in simulated biological matrix. Triamcinolone Acetonide was used as the internal standard (IS). A one step liquid–liquid extraction with ethyl acetate was used for sample cleanup. A gradient elution on a $C_{-18}$ column with acetonitrile and 0.1% o-phosphoric acid in water was employed to get a cleanup baseline and efficient separation of the three chemical entities. Dexamethasone and dexamethasone 21-acetate were observed at 254 nm and paclitaxel was observed at 228 nm. The method was highly selective and the calibration curves were linear over the range of 0.25–10.0 μg/mL with a 12 min chromatographic run time. The assay method was validated in terms of accuracy, precision, absolute recovery, freeze-thaw stability, bench top stability, and reinjection reproducibility. The lower limit of quantitation was 0.25 μg/mL for all the analytes. The intra and inter-batch precision and accuracy were found to be well within acceptable limits (<15%). The assay is simple, reliable, and fast and is deemed suitable for

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application to in vitro release rate studies of these drugs from implantable devices, as well as to their in vivo pharmacokinetic studies.

**Keywords:** HPLC, Assay validation, Dexamethasone, Dexamethasone 21-acetate, Paclitaxel, Simulated biological matrix



## INTRODUCTION

Glucocorticoids have been long used for their potent anti-inflammatory and immunosuppressive properties. Some of the most commonly prescribed glucocorticoids for systemic use include prednisolone, methylprednisolone, triamcinolone, and dexamethasone.[1] Dexamethasone shows the highest anti-inflammatory activity among these steroids and is used systemically to treat acute and severe inflammatory, immunological, and allergic disorders.[2] However, these drugs are also associated with some unwanted side effects on long term systemic use. Hence, localized drug delivery is a favorable option for such drugs. One of the recent uses of dexamethasone, owing to its immunosuppressive action, is in implantable devices such as stents. Delivering dexamethasone from the stents itself rather than by oral administration is useful to minimize the systemic side effects. The prodrug dexamethasone 21-acetate can be coated on the stents.

Paclitaxel is another drug that is used for the prevention of restenosis of stents. Originally used as an anticancer agent, paclitaxel is a novel anti microtubule agent isolated from the bark of the Pacific yew tree, *Taxus brevifolia*. Paclitaxel has been administered for the treatment of breast cancer, ovarian cancer, non small cell lung cancer, and melanoma.[3–5] Recently, paclitaxel has been used to prevent the occurrence of restenosis after angiographic procedures. Dexamethasone eluting stents have been used in clinical studies to evaluate its effect on restenosis after stent implantation.[6–9] Similar clinical trials involving paclitaxel eluting stents have also been conducted to study the effect of paclitaxel in restenosis prevention.[10,11] Both these drugs have resulted in better angiographic outcomes. The clinical studies suggest that dexamethasone and paclitaxel may play an important role in the inhibition of the polymer induced inflammation in this era of drug eluting stents. There are commercially available drug eluting stents (DES) with dexamethasone (Dexamet® Abbott Laboratories) and paclitaxel (Taxus® Boston Scientific).

However, these drugs have been used separately in these DES. In order to improve efficacy, it might be possible to design stents eluting two or more drugs with different mechanisms of action at the same time. With multiple drugs incorporated in the DES, the effect of one drug on the release of the other needs to be assessed. The simple HPLC assay described here is developed for conducting the release rate studies of dexamethasone-21-acetate and paclitaxel (Figure 1) from polymer coated endoluminal stents.

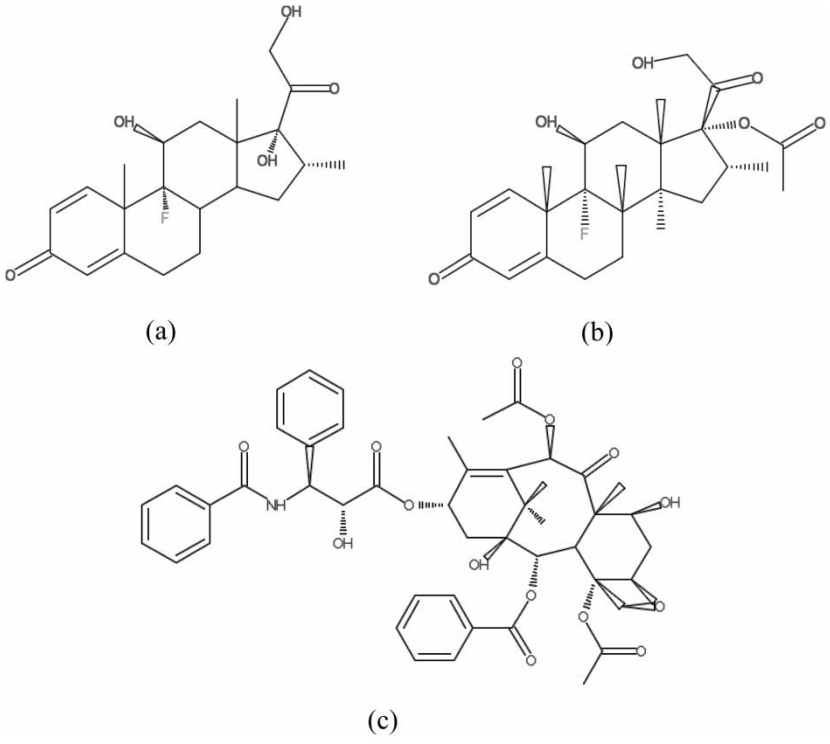


***Figure 1.*** Chemical structures of (a) dexamethasone, (b) dexamethasone 21-acetate, and (c) paclitaxel.



Upon release, dexamethasone-21-acetate, the prodrug used in these stents is converted to the active moiety dexamethasone making it imperative to monitor dexamethasone as well. A simple, sensitive, and economical analytical technique is therefore required for the simultaneous detection of these drugs to assess the performance of the DES designed.

There are numerous reports on LC-MS and LC-MS-MS assays for the estimation of paclitaxel from various biological matrices.[12,13] Most of these assays have been employed for *in vivo* pharmacokinetic or tissue distribution studies of paclitaxel. Though other analytical techniques have also been employed,[14] HPLC methods provide an economical means for the satisfactory determination of paclitaxel in the plasma and other biomatrices.[15–20] Some of these methods showed high sensitivity and selectivity. Similarly a number of HPLC assays have been published in the past for dexamethasone as well. Many of these methods involve solid phase extraction, chemical derivitization, or cumbersome sample cleanup procedures.[21–25]

Mass spectroscopic assays are generally highly sensitive and selective as compared to the HPLC-UV methods but are rather expensive. Hence, for the

present release rate studies we opted for a cost effective HPLC-UV approach. Though assays on both dexamethasone and paclitaxel are widely reported separately, there are no reports to date for their simultaneous estimation using HPLC-UV. Therefore, it was necessary to develop and validate a simple, precise, and reproducible assay method for the quantification of dexamethasone, its prodrug dexamethasone-21-acetate, and paclitaxel for evaluating the performance of DES designed in house.

## EXPERIMENTAL

### Chemicals

Paclitaxel was obtained from LC Laboratories, Woburn MA USA. Dexamethasone, dexamethasone 21-acetate, triamcinolone acetonide, and bovine serum albumin (BSA) were obtained from Sigma, USA. HPLC grade acetonitrile (ACN), ethyl acetate, and phosphoric acid were purchased from Fisher Scientific, USA. Triple distilled water was obtained from an in house water distillation unit. All other chemicals used were obtained from Fisher Scientific, USA and were of analytical grade.



### Equipment

The analysis was performed on an Agilent 1100 HPLC system (Agilent Technologies, Inc. USA) comprised of an Agilent 1100 series thermostated auto sampler, quaternary pump, degasser, and UV-DAD-detector. Data acquisition and analysis were performed using HP chemstation Software Rev A.10.01 run on an IBM computer.

### Chromatographic Conditions

Chromatographic separation of the analytes and internal standard were achieved using gradient elution on a $C_{-18}$ column (3.5 μm, 4.6 × 50 mm, Waters' Symmetry®) fitted with a precolumn filter at 1.25 mL/min flow rate. Mobile phase consisted of 5% acetonitrile in water as Solvent A and 80% acetonitrile in water (0.1% phosphoric acid) as solvent B, respectively.

During each run solvent, B was increased from 35% to 70% in 6 minutes and then brought back to the initial conditions. The run time was 12 minutes. The auto sampler was set at 4°C. The analysis was performed at room temperature 25°C (±2°C) and the UV detector was set at 254 nm and 228 nm.

## Sample Cleanup Procedure

The sample cleanup involved a simple, single step liquid-liquid extraction (LLE) with ethyl acetate. To 200 μL of bio-matrix (or blank, calibration standards, or quality control standards) 10 μL of IS was added in an eppendorf tube. This mixture was extracted with 1000 μL of ethyl acetate on a vortex mixer for 60 seconds. This was followed by centrifuging the samples at 8000 rpm for 10 min. The organic layer was transferred to a glass tube and dried in a speed vacuum concentrator (Jouan Inc). The dry residue was reconstituted in 200 μL of reconstitution solution (a 1:1 mixture of solvents A and B) and 50 μL of this was used for injecting onto the HPLC column.



## Standard Solutions

The primary stock solutions (1 mg/mL) of dexamethasone and dexamethasone 21-acetate were prepared in methanol while paclitaxel stock solution was prepared in acetonitrile. The primary stock solutions were diluted with acetonitrile to get working stock solutions of 25 (WS-1), 100 (WS-2), and 500 μg/mL (WS-3) concentrations. These working stock solutions were used to prepare the analytical standards (AS), calibration standards (CS), and quality control samples (QC).

The analytical standards at 0.25, 0.5, 1, 2, 5, and 10 μg/mL were prepared by diluting the working stock solutions with reconstitution solutions. A solution of BSA in PBS (4.4 g per 100 mL) was used as the simulated bio-matrix for the present study for preparing CS and QCs. Quality control samples at the concentrations of 0.5, 2, and 10 μg/mL as low, medium, and high concentrations were also prepared similarly.

Triamcinolone acetonide was used as the internal standard. The standard and quality control samples were spiked with 10 μL of IS working stock solution (100 μg/mL) to yield a final concentration of 1 μg/mL.

## Method Validation

The assay method was validated for three days in terms of system reproducibility and selectivity, lower limit of quantitation (LLOQ), limit of detection (LOD), linearity, absolute recovery, accuracy and precision, freeze thaw stability, dry residue stability, and bench top stability.

### Linearity, Limit of detection, Limit of Quantitation

The linearity for each analyte was checked with calibration curves obtained by linear least square regression. The calibration curves were weighted according

to $Y^{-n}$, such that $n = 0$, 1, or 2 and Y is the observed peak area ratio. Residual analysis indicated that the weighting factor of $(1/Y^2)$ provided the best fit of the calibration curves. The LOD was defined as the lowest concentration of the analyte that had an S/N ratio $> 3$. The LLOQ was defined as the lowest concentration of the analyte that could be analyzed with predetermined accuracy and precision.[26]

### Recovery, Accuracy and Precision

The recovery of each of the analytes was determined by comparing the peak area ratios of the analytes to IS after liquid-liquid extraction to those from the analytical standards.

The accuracy and precision for the method was determined by using the quality control samples, (low, medium, and high) run in five replicates on 3 different days and were reported in terms of % bias. This was calculated for intra-day as well as inter-day deviation. This % bias was calculated as follows:

$$\%\text{Bias} = (\text{observed conc.} - \text{nominal conc.}) \times 100/\text{nominal conc.}$$

A one way analysis of variance (ANOVA) was used to calculate the inter and intra batch precision in terms of % RSD.



## RESULTS AND DISCUSSION

### Method Development

The sample cleanup procedure had to be optimized to suit the three analytes and the internal standard. Liquid-liquid extraction (LLE) procedures with various solvents like t-butyl methyl ether, ethyl actetate, and methylene chloride were widely reported in literature for dexamethasone and paclitaxel.[27–29] Hence, sample cleanup procedures using different organic solvents were tried to improve the recovery and to minimize interference from the matrix. Use of t-butyl methyl ether gave an unknown peak that interfered with the analytes and the absolute recovery was only about 35% for all the analytes. Therefore, ethyl acetate was tested and was found to improve the recoveries to about 55% for dexamethasone and dexamethasone 21-acetate and 45% for paclitaxel with consistent reproducibility, and there were no interfering peaks. Hence, ethyl acetate was selected for the present assay method.

During the LC development, isocratic as well as gradient flow was tested. The isocratic run was unable to separate the three analytes under consideration. Also various columns were used and a $C_{-18}$ column gave the best separation with gradient flow. The gradient flow was adjusted further to prevent the interference of an impurity peak (Figures 2–3). At the current flow rate and gradient, excellent separation was observed with good peak shapes. No other endogenous

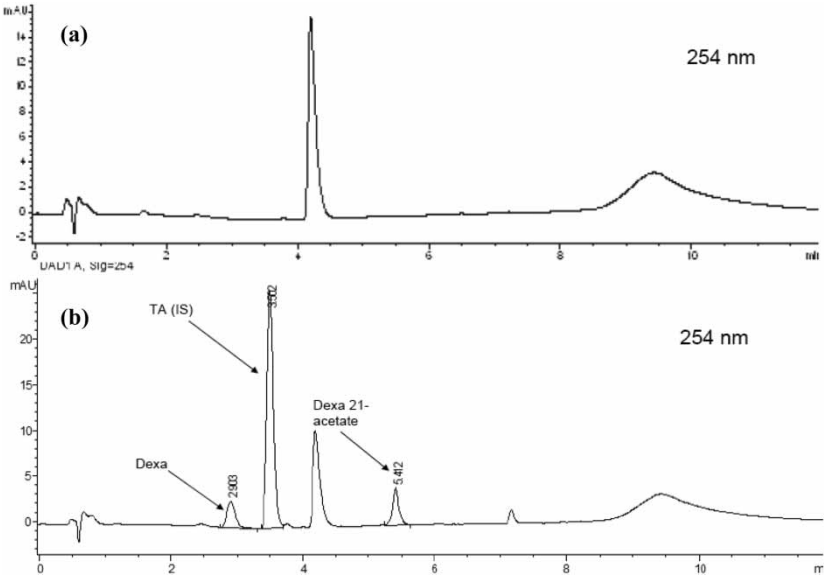


*Figure 2.* Representative chromatograms at 254 nm showing specificity of the assay, (a) processed blank simulated bio-matrix and (b) bio-matrix spiked with 0.50 μg/mL of dexamethasone and dexamethasone 21-acetate.

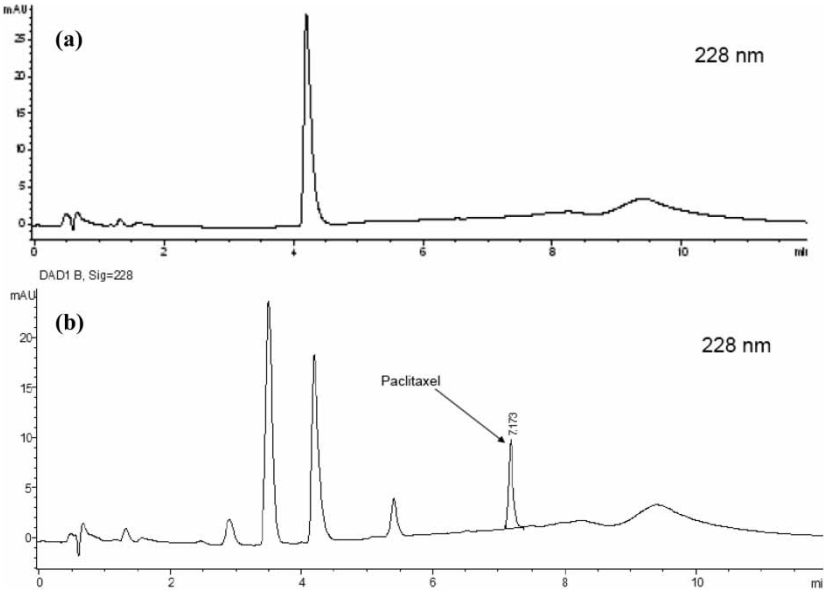


*Figure 3.* Representative chromatograms at 228 nm showing specificity of the assay, (a) processed blank simulated bio-matrix and (b) bio-matrix spiked with 0.50 μg/mL of paclitaxel.

or exogenous peaks were observed interfering with the assay. Dexamethasone and dexamethasone 21-acetate showed better absorbance at 254 nm and that for paclitaxel was at 228 nm. Thus, the analytes were simultaneously detected at two different wavelengths using the diode array detector (DAD).

## Assay Validation

The system reproducibility was assessed by injecting each of the analyte standards in triplicate. The variation in the peak area ratios for each of the three analytes was less than 5% at all concentration levels. This indicated that the system yielded reproducible data. Absence of interfering peaks in the region of interest is defined as specificity of the assay method. This was verified by observing the chromatograms of blank BSA solution, BSA solution spiked with internal standard, and analytes. The chromatograms are shown in Figures 2–3.



## Linearity, Limit of detection, Limit of Quantitation

The calibration curves on the three days of validation were linear over the concentration range of 0.25–10.0 μg/mL for each of the analytes. The LOD for dexamethasone and dexamethasone 21-acetate was found to be 0.15 μg/mL and that for paclitaxel was 0.1 μg/mL. The LLOQ for all analytes was 0.25 μg/mL (%CV $<$ 20). The slope, intercept, and the coefficient of determination values for the calibration curves are given in Table 1. The retention times (RT) for dexamethasone, dexamethasone 21-acetate, paclitaxel, and internal standard triamcinolone acetonide were 2.9, 5.4, 7.2, and 3.5 min, respectively.

## Accuracy and Precision

The results for the accuracy and precision parameters are displayed in Table 2. The intra and inter-batch variability were within the acceptable limits of

***Table 1.*** Summary of linearity parameters for the calibration curves ($y = mx + c$) during assay validation

| Analyte | $r^2$ | Slope[a] | Intercept[a] |
|---|---|---|---|
| Dexamethasone | 0.99 | 0.2816 ± 0.007 | −0.0055 ± 0.001 |
| Dexamethasone 21-acetate | 0.99 | 0.2381 ± 0.008 | 0.0171 ± 0.001 |
| Paclitaxel | 0.99 | 0.4054 ± 0.021 | 0.0305 ± 0.005 |

[a]Mean ± SD, N = 3.

*Table 2.* Quality control report: assessment of accuracy and precision of the method

| Analyte | QC Samples (μg/mL) | Accuracy (%bias) | | Precision (%RSD) | |
|---|---|---|---|---|---|
| | | Inter-batch | Intra-batch | Inter-batch | Intra-batch |
| Dexamethasone | 0.50 | 3.51 | 4.73 | 9.97 | 5.72 |
| | 2.00 | 3.23 | 4.70 | 8.21 | 4.64 |
| | 10.00 | 3.46 | 2.88 | 9.15 | 4.37 |
| Dexamethasone 21-acetate | 0.50 | 6.67 | 7.37 | 0.82 | 6.82 |
| | 2.00 | 3.65 | 4.22 | 3.04 | 5.26 |
| | 10.00 | 4.15 | 4.81 | 5.60 | 4.11 |
| Paclitaxel | 0.50 | 6.64 | 6.62 | 5.92 | 3.41 |
| | 2.00 | 1.92 | 1.55 | 11.70 | 7.59 |
| | 10.00 | 4.21 | 3.63 | 3.41 | 5.70 |



±20% at low and ±15% at all other concentrations. The assay method was thus found to be accurate and precise over the concentration range studied.[26]

## Stability Studies

Quality control samples were subjected to short term room temperature, long term storage conditions (−70°C), and freeze thaw stability (3 cycles) studies. All the stability studies were carried out at three different concentration levels (0.5, 2, and 10 μg/mL as low, medium, and high, respectively) in triplicate. There was less than 15% deviation between the responses of spiked standards at time zero and after 8 hours for all the three analytes at room temperature. This indicated that the analytes are stable at room temperature for over 8 hrs, which well encompasses the duration of typical sample handling and processing. The assay was also checked for reinjection reproducibility to determine if an analyte run could be reanalyzed in case of an unexpected delay in analyses. The same set of QC samples was repeated after one injection with a gap of 4 hours, during which they were stored at 4°C. The results for these studies are presented in Table 3. In all cases, the deviations were less than 10%. QC samples were stored at −70°C for 7, 15, and 30 days and analyzed. The data in Table 3 indicates that the deviation is less than 10% demonstrating the stability of the samples on long term storage at −70°C.

It is evident from Figure 4 that the deviations after the first, second, and third freeze thaw cycle are not greater than 10% at all concentration levels. This indicates adequate freeze thaw stability. Quality control samples at low, medium, and high concentrations in triplicate were used for this purpose. The quality control samples were extracted and analyzed immediately, and used as the reference point to determine the percentage deviations

***Table 3.*** Stability data for the three analytes (n = 3)

| Analyte | Storage conditions | Nominal Conc. (μg/mL) | Mean Conc. at t = 0 (μg/mL) | Mean Conc. recovered (μg/mL) | SD (μg/mL) | RSD (%) |
|---|---|---|---|---|---|---|
| Dexamethasone | 8 hours at 25°C | 0.50 | 0.50 | 0.52 | 0.01 | 1.81 |
| | | 2.00 | 2.04 | 2.19 | 0.03 | 1.45 |
| | | 10.00 | 10.00 | 10.13 | 0.12 | 1.20 |
| | 30 days at −70°C | 0.50 | 0.50 | 0.47 | 0.05 | 9.94 |
| | | 2.00 | 2.04 | 2.05 | 0.05 | 2.24 |
| | | 10.00 | 10.00 | 10.46 | 0.16 | 1.54 |
| Dexamethasone 21-acetate | 8 hours at 25°C | 0.50 | 0.54 | 0.50 | 0.02 | 4.00 |
| | | 2.00 | 2.06 | 1.87 | 0.01 | 0.53 |
| | | 10.00 | 10.30 | 9.40 | 0.34 | 3.67 |
| | 30 days at −70°C | 0.50 | 0.54 | 0.52 | 0.01 | 1.84 |
| | | 2.00 | 2.06 | 2.06 | 0.01 | 0.70 |
| | | 10.00 | 10.30 | 11.26 | 0.15 | 1.29 |
| Paclitaxel | 8 hours at 25°C | 0.50 | 0.52 | 0.49 | 0.02 | 4.48 |
| | | 2.00 | 2.09 | 2.03 | 0.01 | 0.52 |
| | | 10.00 | 10.24 | 9.49 | 0.50 | 5.25 |
| | 30 days at −70°C | 0.50 | 0.52 | 0.47 | 0.01 | 0.25 |
| | | 2.00 | 2.09 | 2.06 | 0.04 | 2.02 |
| | | 10.00 | 10.24 | 10.51 | 0.24 | 2.24 |

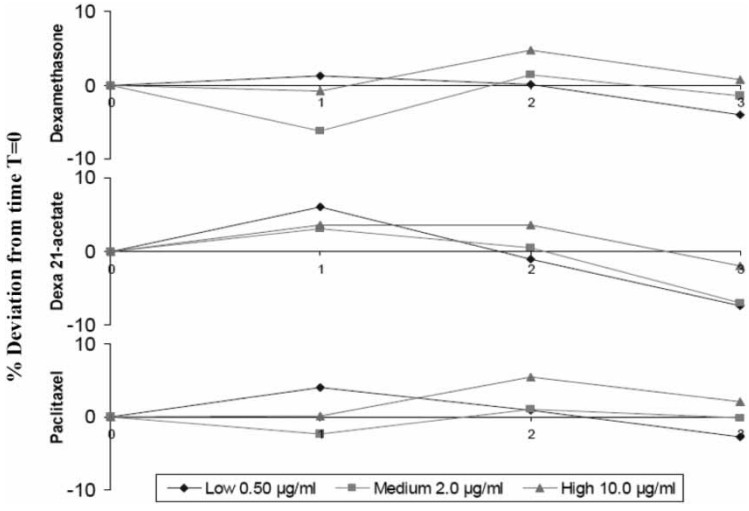


***Figure 4.*** Freeze thaw cycle stability data for the analytes at three concentration levels. X-axis represents number of ft cycles.



after each freeze thaw cycle. Similarly, the stability of the extracted dry residue was established with less than 10% deviation.

## Human Plasma

The assay was applied to human plasma samples spiked with these drugs and calibration curves along with quality control samples were run. The assay was linear over the concentration range of 0.25–10.0 μg/mL range, similar as that in simulated biomatrix. No interfering peaks were observed with the human plasma samples. This indicates that the method can be applied directly, without any modification for the analysis of these drugs in human plasma samples as well. The absolute recoveries of the dexamethasone and dexamethasone 21-acetate in plasma were about 45%, while that of paclitaxel was about 38%. The recoveries were thus comparable to the simulated matrix used.

There is no published analytical method to simultaneously determine dexamethasone 21-acetate, dexamethasone, and paclitaxel in biomatrix to the best of our knowledge. Since the scope of the current experiment was to study the release rate characteristics of these multiple drugs from various polymer coatings from stent surfaces, a simulated biomatrix of BSA media was used. Maintaining the BSA concentration in the media similar to plasma sufficed the current study needs of studying the release rates.

## CONCLUSION

A simple and precise HPLC method for the simultaneous quantification of dexamethasone 21-acetate, dexamethasone, and paclitaxel was developed and fully validated. This method offers added advantages of faster run time and lower sample requirements. The present quantitation limit (0.25 μg/mL) can be further improved, if required by sample concentration. The results of assay performance indicate that the method is precise and accurate for the routine determination of dexamethasone 21-acetate, dexamethasone, and paclitaxel simultaneously. The assay was also applied to human plasma by spiking human plasma with the standards. Hence, the method can be considered suitable for application to release rate studies from stents, preclinical as well as clinical pharmacokinetic studies. The assay can be used to obtain the concentration time profiles of dexamethasone 21-acetate, dexamethasone, and paclitaxel for the realistic estimation of their pharmacokinetic parameters.



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