Development of a Method for Determination of Dexamethasone (DEX) Concentrations in Human Plasma by Ultra Performance Liquid Chromatography / Photo Diode Array Detector (UPLC/PDA)

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ABSTRACT

Objective: At present, Cushing's syndrome (CS) is diagnosed by measuring cortisol levels and the dexamethasone suppression test. However, the false positive results were found in patients who have stress or lack of drug compliance. Several studies reported a relationship between cortisol and dexamethasone concentration in plasma and measurement of both has proved most useful for identifying patients' diagnoses. Although, many highly sensitive and specific methods have been reported, the method which was suitable for routine analysis was required. In this study, we aimed to develop a cheaper UPLC-PDA method and to fully validate it for determination of dexamethasone in human plasma.

Methods: Chromatographic separation was carried out by the ACQUITY UPLC™ BEH Shield RP, 1.7 μm (100 x 2.1 mm.I.D.) with mobile phase consisting of 0.05% Trifluoroacetic acid / Acetonitrile in gradient program. The Photodiode Array (PDA) Detector was set at 240.2 nm.

Results: The run time was 6 min, 2.40 min for dexamethasone and 3.67 min for prednisolone (IS). The limit of quantification was 2.0 ng/mL. The standard curves are in the range 2-60 ng/mL with good linearity ($r^2 > 0.9975$). The method was acceptable in terms of selectivity, accuracy, precision, stability and matrix effect. The measured dexamethasone concentrations ranged from 2.0 - 8.2 ng/mL (5.12 - 21.03 nmol/L).

Conclusion: A highly rapid, sensitive, accurate and reproducible UPLC-PDA method was developed and fully validated for the determination of dexamethasone in plasma. This method was successfully applied to routine measurement of dexamethasone concentration in plasma of patients after the low-dose dexamethasone suppression test.

Keywords: UPLC/PDA, dexamethasone, Cushing's syndrome

INTRODUCTION

Cortisol is a steroid (glucocorticoid) hormone produced by the adrenal gland. It is the essential hormone for life which used to control blood sugar, immune system, anti-inflammatory system, balance of electrolyte and function of central nervous system. Cortisol is released from adrenal cortex by activation of hypothalamus and pituitary gland in brain. Abnormal increase in cortisol level is diagnosed as Cushing's syndrome (CS). In contrast, abnormal decrease in cortisol level is diagnosed as Addison's disease.[1]

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At present, Cushing's syndrome (CS) is diagnosed by analysis of a 24-hour urine cortisol and low-dose dexamethasone suppression test (LDDST) by measuring how cortisol levels change after taking one milligram dose of dexamethasone at night and in the morning time.[2,3] However, the reported sensitivity of this test is 98-100% and specificity is 87.5%.[3-5] The false positive results were found in non- Cushing's syndrome patients such as fat, stress, alcoholism, high cortisol binding globulin, glucocorticoid resistance or lack of drug compliance.[3] The false positive results lead to unnecessary testing and patients often end up by wasting even more time and money.

Several studies reported a relationship between cortisol level and dexamethasone concentration in plasma and measurement of both has proved most useful in identifying patients.[6,7] Moreover, Meikle reported the cut off of dexamethasone concentration should be more than 5.6 nmol/L (2.18 ng/mL) in the evaluation for false positive results.[6]

Now, the general laboratory can analyze cortisol level by automated machine but few laboratories can analyze dexamethasone concentration by complicated chromatographic machine. As a low dose of dexamethasone is taken in the dexamethasone suppression test, many highly specific and highly sensitive methods have been reported for determination.[8-16] The most common is LC-MS/MS method[8-11,16] which can measurement dexamethasone at the low concentration. However, this method is more difficult, expensive, and time consuming than UPLC method,

and is not suitable for routine analysis. UPLC with photodiode array detection is regular employed for drug determination, the parameter in identifying a drug are its retention time and UV spectrum. This easier and cheaper method is not only highly specific and sensitive but also suitable for routine analysis. Efficiency of analysis is of great importance in many applications, especially in bio-analysis. In this study, we aimed to develop and validate our UPLC-PDA method for determination of dexamethasone in human plasma.

MATERIALS AND METHODS

1. Chemicals
Dexamethasone and Prednisolone were purchased from Sigma-Aldrich Ltd. (Steinheim, Germany), and the chemicals's structure are as shown in Fig 1. The HPLC grade acetonitrile and methanol (MeOH) were purchased from Labscan Ltd. (Bangkok, Thailand). Milli-Q water from water purification system, Millipore Corporation (Massachusetts, USA) was used. Other chemicals were of analytical grade. Drug-free human plasma from the Department of Transfusion Medicine, Siriraj Hospital was used and the protocol was approved by Siriraj Institution Review Board, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

2. Instrumentations
Acquity™ Ultra Performance Liquid Chromatography from Waters Co., Ltd. (USA) was used for separation module. Separation was achieved on the ACQUITY UPLC™ BEH Shield
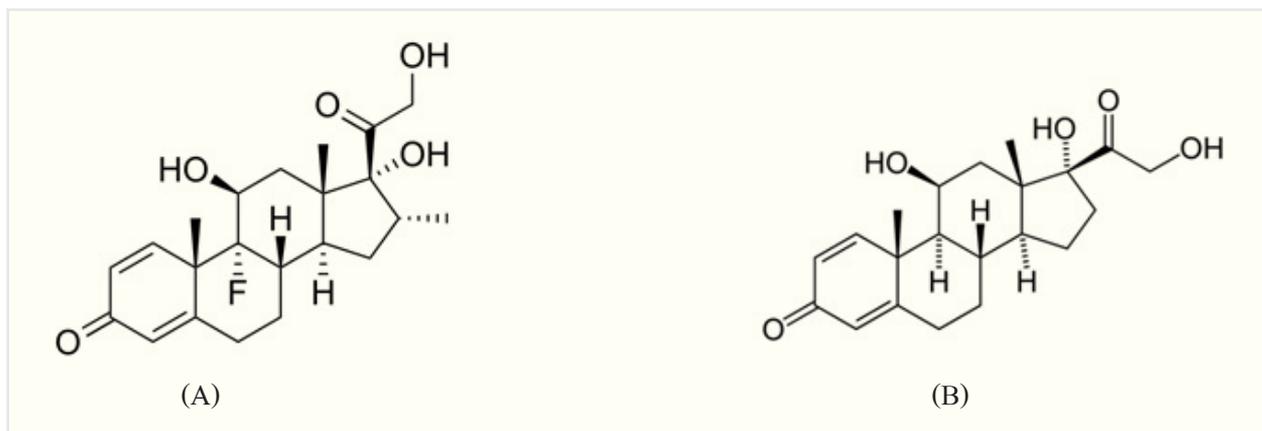

Fig 1. The chemical structure of (A) dexamethasone, (B) prednisolone (IS) from Frerichs VA, et al. (2004)[10]

RP, 1.7 μm (100x2.1 mm.i.d.) from Waters Co., Ltd. (USA). The ACQUITY UPLC® Photodiode Array (PDA) Detector from Waters Co., Ltd. (USA) was operated at wavelengths between 200-380 nm and quantification was done at 240 nm. The Empower 2 software was used for data management.

3. Standard stock solutions

Standard stock solutions of Dexamethasone and Prednisolone (internal standard) were prepared in MeOH and this working standard was diluted with milli-Q. The stock solutions of dexamethasone were diluted to concentrations which ranged from 100-3,000 ng/mL which were used as working solutions. The Quality Control (QC) samples were prepared at 100, 300, 1,250, and 2,500 ng/mL. Standard solutions were stored at -20 degree Celsius until use.

4. Sample preparations

The liquid-liquid Extraction (LLE) was used for sample preparation. One mL aliquot of the spiked plasma and 20 μL of 2.5 μg/mL prednisolone (IS) were pipetted into a 2 mL centrifuge tube. The mixture was protein precipitated with 500 μL of acetonitrile, the samples were mixed for 10 min and centrifuged at 4,000 rpm for 10 min. Then, 1,000 μL of supernatant were transferred into the 2 mL centrifuge tube. The samples were extracted with 1 mL of diethyl ether and 120 μL of ammonia solution, mixed for 10 min and centrifuged at 4,000 rpm for 10 min. After that, the organic layers were transferred into a 1.5 mL centrifuge tube and evaporated to dryness. The residue was reconstituted with 120 μL of mobile phase before it was injected into the UPLC system.

5. Bio analytical method validation

The developed method was fully validated according to the USFDA guidance.[17]

5.1 Selectivity and sensitivity

The selectivity was examined using six sources of free drug plasma which were extracted and analyzed by the developed method. The sensitivity at the limit of quantification (LOQ) was also examined on the basis of signal to noise ratio of 5:1. LOQ and should be reproducible with the percentage of coefficient of variation (%CV) within ± 20%.

5.2 Accuracy and precision

Accuracy and precision were examined by six replicate analyses of plasma spiked with four different concentrations (LOQ, 6, 25 and 50 ng/mL) for three separate days. The precisions of each method were calculated as the percentage of coefficient of variation (%CV) which should be within ± 20% at LOQ and 15% at other concentrations. The accuracy was calculated as percentage of relative error (%RE) which should be in the range 80-120% at LOQ and 85-115% at other concentrations.

5.3 Linearity and calibration curve

A calibration curve was assessed by a linear regression model, $y=mx+b$ and weighted by $1/x$, where y is the ratio of peak area of analyte to the peak area of IS, and x is the concentration at 2, 10, 20, 30, 40 and 60 ng/mL. The coefficient of determination (r^2) should be more than 0.995.

5.4 Recovery of extraction

The recovery of method was performed by comparing peak areas of the extracted samples at 6, 25 and 50 ng/mL with peak areas of non-extracted standard solution at the same concentrations. The percentage of absolute recovery (% RV) was the ratio of the measured extracted peak area to the non-extracted peak area.

5.5 Stability

The stability of analysis was performed by three replicated analyses of plasma spiked at 6, 25 and 50 ng/mL under various conditions including 3 cycles of freeze and thaw, short term stability, post-preparative stability and long term stability. The acceptable percentage of variation must be within ±15%.

5.6 Matrix effect

Matrix effects were determined in terms of matrix factor (MF). MF is the ratio of the peak responses in the presence of matrix to peak response in the absence of matrix. The absolutely acceptable matrix effect should be within 0.8-1.2

which means no matrix effect on analysis by this developed method.

6. Bio-analysis

This method was used to determine concentrations of dexamethasone in plasma of 30 patients after LDDST. The protocol was approved by Siriraj Institutional Review Board, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

RESULTS

The mobile phase was optimized in a gradient program for the determination of dexamethasone. A mixture of 0.05% Trifluoroacetic acid / Acetonitrile was used in this gradient program, and 25/75(v/v) at initial, 32/68(v/v) at 4 min and 25/75(v/v) at 6 min was found to be the most suitable. This method has an excellent separation of dexamethasone and internal standard which were achieved within 6 min. The appropriate retention time were 2.4 min for IS and 3.67 min for dexamethasone. A chromatogram showed the separation of IS and dexamethasone. The capacity factor (k') was 2.99 for IS and 5.12 for dexamethasone while the resolution factor was 20.15. The PDA spectrums of individual drugs have been shown in Fig 2 and the optimum wavelength was selected at

240.4 nm which had much better detector response for dexamethasone.

No interference peak was observed at the retention time of both prednisolone and dexamethasone as shown in Fig 3. The limit of quantification (LOQ) was found to be 2 ng/mL with the coefficient of variation of 5.75% and the relative error was 110.0%. The calibration cure was assessed by plotting the ratio of peak area of dexamethasone to the peak area of IS versus concentration in the range of 2 – 60 ng/mL. This method has a good linearity with the coefficient of determination (r^2) more than 0.997 as shown in Fig 4. All were within the acceptable range ($r^2 > 0.995$). This method has acceptable precision and accuracy as summarized in Table 1. Within-day precision of the method was found to be in the range of 3.79-13.09% and accuracy ranged from 89.10-110.00%. Between-day precision of the method was found to be in the range of 4.95-10.89% and accuracy ranged from 91.31-103.89%. The absolute recovery of extraction (%RV) was found to be in the range of 56.11-76.37% which was reproducible as shown in Table 2. This study show that the plasma samples containing dexamethasone were stable in various tested conditions as demonstrated in Table 3. The percentage of variation in each condition was within the acceptable range. No matrix effect was observed when a matrix effect test was
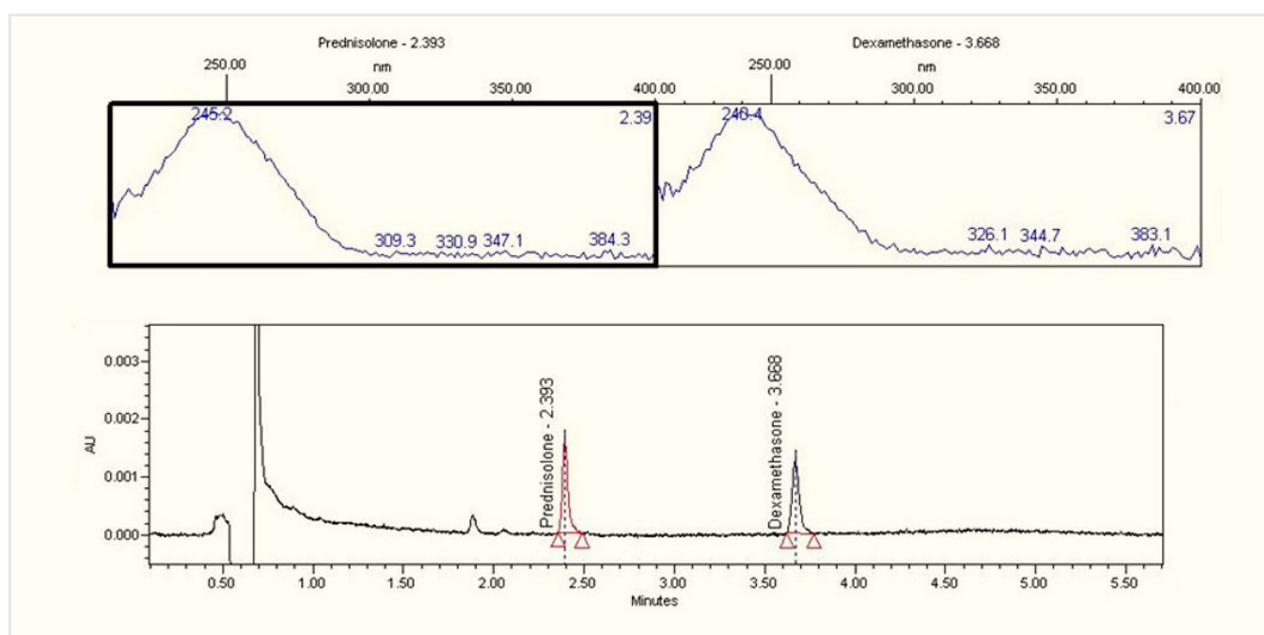

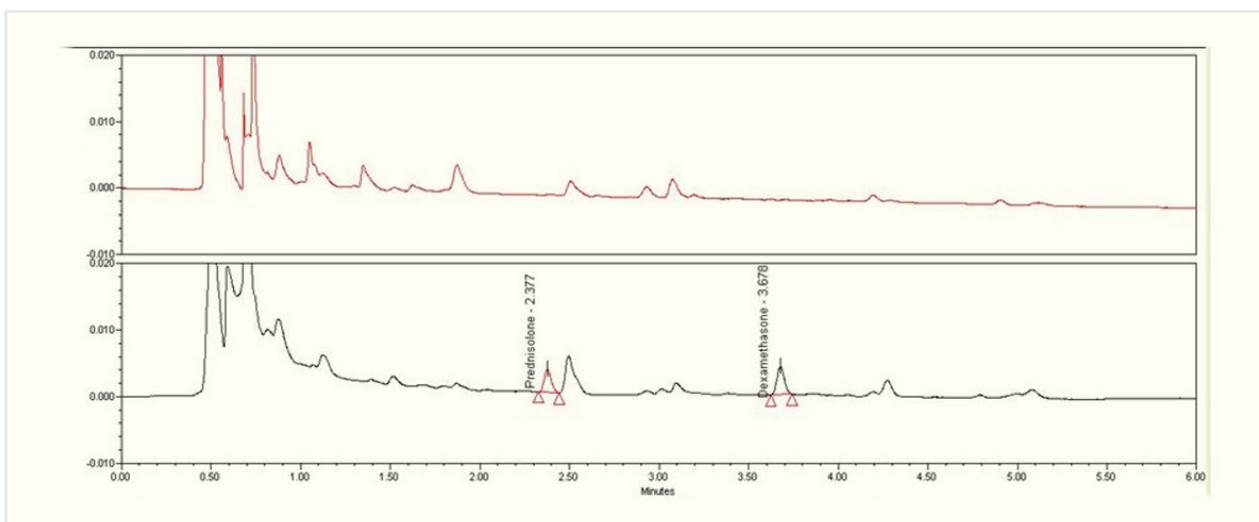**Fig 2.** The PDA spectrum of prednisolone (IS) and dexamethasone.

Fig 3. The chromatograms of extracted (A) blank plasma, (B) plasma spiked with 50 ng/mL of dexamethasone and IS.
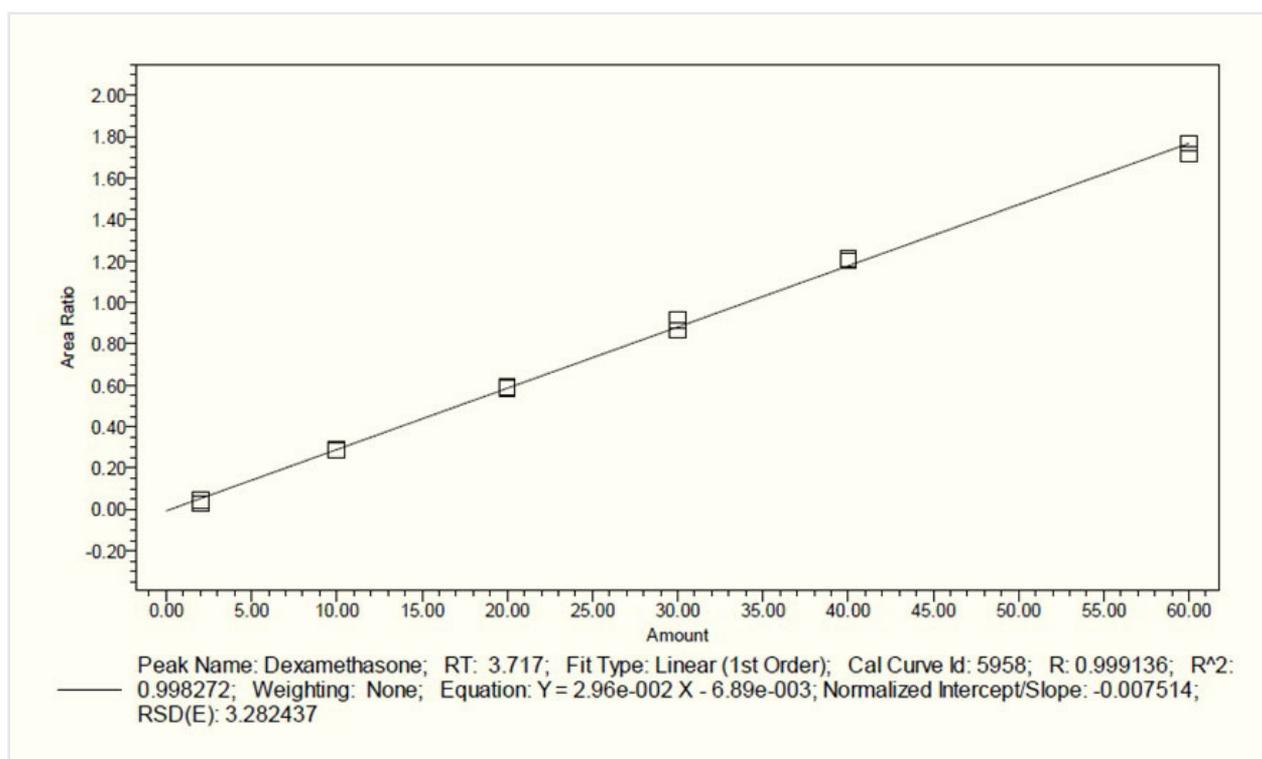

Peak Name: Dexamethasone; RT: 3.717; Fit Type: Linear (1st Order); Cal Curve Id: 5958; R: 0.999136; R^2: 0.998272; Weighting: None; Equation: Y = 2.96e-002 X - 6.89e-003; Normalized Intercept/Slope: -0.007514; RSD(E): 3.282437

Fig 4. The calibration curve of dexamethasone.

TABLE 1. The precision and accuracy (within-day and between-day).

Expected concentration (ng/mL)	Within day (n=6)			Between days (n=18)		
	Mean ± S.D. (ng/mL)	Precision (%CV)	Accuracy (%RE)	Mean ± S.D. (ng/mL)	Precision (%CV)	Accuracy (%RE)
2	2.2±0.13	5.75	110.00	2.08±0.23	10.89	103.89
6	5.83±0.31	5.27	97.22	5.97±0.33	5.45	99.44
25	24.58±0.93	3.79	98.33	25.33±1.31	5.19	101.33
50	45.62±2.93	6.43	91.23	45.66±2.26	4.95	91.31

n = number of replicates, % CV = percentage of coefficient of variation

%RE = percentage of relative error

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performed. The matrix factors were 0.99-1.02 and 1.04 for dexamethasone and IS, respectively.

Dexamethasone concentrations in plasma of 30 patients after LDDST were determined. The dexamethasone concentrations ranged from 2.0-8.2 ng/mL which associated with low dose of dexamethasone ingestion. However, the concentration of three patients was less than 2.0 ng/mL which may be variation of absorption and metabolism of the dexamethasone.

DISCUSSION

The developed method was fully validated according to the USFDA guideline. The validated result indicated that this method was highly sensitive, precise and accurate to determine dexamethasone. The applied chromatography condition of UPLC permitted a good separation even though the structure of dexamethasone is closely related with prednisolone (IS). PDA detection is used for drugs identification in combination with retention times.

In comparison with other published methods, our UPLC method was rapid and the run time was within 6 min with a dramatic reduction in retention time from more than 10 to 3.67 min.[14,16] The limit of quantification of this study was 2 ng/mL (5.12 nmol/L) which was reproducible and less than the cut-off level (5.6 nmol/L) of the dexamethasone concentration after LDDST reported by Meikle.[6] Therefore, this concentration was accepted as the limit of quantification.

For the extraction, the previously published methods used complicated and high cost solid phase extraction for sample extraction.[9-11,14-16] In this experiment, the liquid-liquid with diethyl ether and ammonia solution was used because it was an easier extraction method with lower cost. The recoveries of extraction were acceptable with repeatability, even though it requires a larger sample volume (1.0 mL) than the previously published methods (0.2-0.5 mL).[9,10,15,16] After that, the stability of dexamethasone in plasma was examined and the result indicated that dexamethasone had a good stability in human plasma and also in auto sampler.

Our developed method was applied to determine dexamethasone concentrations in plasma of patients with suspected Cushing's syndrome and the detection of the concentrations was between 2.0-8.2 ng/mL (5.12-21.03 nmol/L). This result was in agreement with previous studies, 3.3-19.6 nmol/L[6] and 3.0-21.5 nmol/L.[11] Moreover, the measurement of dexamethasone is recommended to be measured together with cortisol level in LDODST.

TABLE 2. The recovery of extraction.

Concentration (ng/mL)	Recovery of extraction (%RV)	Precision (%CV)
Dexamethasone		
6 (n=6)	76.56	1.24
25 (n=6)	60.50	0.51
50 (n=6)	54.30	1.37
Prednisolone		
25 (n=6)	55.36	1.93

n = number of replicates

TABLE 3. The stability of dexamethasone (N=3).

Stability test	Variation (%)		
	LQC (6 ng/mL)	MQC (25 ng/mL)	HQC (50 ng/mL)
Freeze and thaw (3 cycles)	-9.89	0.40	-6.62
Short term (6 hr.)	-0.55	-3.49	-8.50
Long term (60 day)	1.15	1.17	5.68
Post-preparative (in auto sampler) 8 hr.)	2.75	-8.59	-8.08

n = number of replicates, LQC = Lower quality control, MQC = Medium quality control, HQC = High quality control

CONCLUSION

UPLC Technology has been adopted successfully in laboratories around the word for the most demanding separation. It is highly robust, dependable and reproducible. The UPLC with photodiode array (PDA) developed method provides a rapid, sensitive precise and accurate analysis for the quantification of dexamethasone in human plasma. All the validation parameters in this study were found to be within the acceptable range of USFDA guideline. This method was successfully applied to routine measurement of dexamethasone concentration. It will be beneficial for the patients who need to determine their dexamethasone level as increasing for suspected Cushing's syndrome.

ACKNOWLEDGMENTS

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