Running Title:

Determination of steroids in urine samples.

Title of the Article:

Simultaneous HPLC Determination of Betamethasone and Prednisolone in Urine samples using Monolithic Column

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Abstract:

An HPLC method is reported for the separation and quantification of betamethasone and prednisolone in urine samples using Chromolith® Performance RP-18e (100 mm x 4.6 mm) column. The separation and detection was achieved with mobile phase composed of methanol: water (44: 56 v/v) at 254 nm.

The method has been validated for the system suitability, linearity, precision and accuracy, limits of detection, specificity, stability and robustness. The limits of detection for the prednisolone and betamethasone are 0.11ng and 0.075 ng per 10µl injection, respectively. The % recovery for spiked urine samples was 97 %- 103 %.

Key words: Monolithic Column, HPLC, Betamethasone, Prednisolone, Urine.

Introduction

Corticosteroids are a family of drugs that include cortisol (hydrocortisone), an adrenal hormone found naturally in the body, as well as synthetic drugs. Though natural and synthetic corticosteroids are both potent anti-inflammatory compounds, the synthetics exert a stronger effect. Corticosteroids derivatives; betamethasone, dexamethazone, prednisolone, triamcinilone including cortisone are used to treat numerous autoimmune and inflammatory conditions, including asthma, bursitis, Crohn's disease, skin disorders, tendinitis, ulcerative colitis and others[1]. Assay of steroids is important in pharmaceutical formulations[2,] and in biological fluids; to disease diagnosis[3], pharmacokinetics [4], study metabolism of selected steroids [5, 6] and as a test for doping and veterinary control [7, 8]. Separation techniques like HPLC [9], GC [10] and CE [11] are reported to separate and determine the multiple steroids and single analyte of interest from its interfering components. To separate the analytes from endogenous materials, use of coupled column chromatography with mass spectrometric [12] or tandem MS is reported [13].

Monolith columns are new generation in HPLC stationary phases. Silica-based monoliths have small-sized skeletons and a bimodal pore size distribution with μm-sized throughpores and nm-sized mesopores. This gives silica-based monoliths favorable properties for high-efficiency, fast separations, like a low-pressure drop across the column, fast mass transfer kinetics and a high binding capacity [14]. Many successful applications are reported in pharmaceutical and biological analysis [2, 15, 16].

Here we attempt to use monolithic column for separation of betamethasone, corticosteroids known for its effects on nervous system, carbohydrate metabolism and cardiovascular (attractive for drug doping) from predinisolone and urinary endogenous compounds[12]. The work will be extended to develop method for

simultaneous determination and screening of blood and urine samples including other corticosteroids.

Material and Methods:

Chemicals

Prednisolone, betamethasone and cortisone were obtained from Pfizer Laboratories Ltd. Pakistan, GSK Pakistan (Ltd) and Sigma–Aldrich, St. Louis, MO, USA respectively. HPLC grade Methanol was purchased from Sigma-Aldrich Chemi GmbH Germany. Water was doubly distilled and deionized. Mobile phase components were degassed before use.

Instrumentation

Spectra SYSTEM P-2000 Pump with a UV6000LP Diode Array Detector and SCM1000 Degasser (Thermo Finigan) was used for the present study.

The separations were achieved with an analytical column Chromolith® Performance RP-18e (100 mm x 4.6 mm), by Merck KGaA (Darmstadt, Germany)) with an isocratic mobile phase of MeOH/H_2O (44 : 56 v/v) at a flow rate of 2.0 ml min^{-1}. Detection was achieved at 254 nm. ChromQuest software was used for data analysis.

Standard Solutions of the Corticosteroids

The stock solutions of 1000 µg mL-1 (1 mg mL^{-1}) of each corticosteroid were prepared in Methanol. The working standard solutions were prepared by diluting aliquots of each stock solution to obtain concentrations ranging from 1 to 10 µg mL^{-1}. The calibration graphs were constructed by plotting the peak areas obtained at wavelength 254 nm versus the corresponding injected amounts (ng).

Urine sample preparation

The urine samples were collected from five volunteers. Steroids were determined by spiking urine samples of healthy persons using solid phase extraction technique. Discovery DSC-18 SPE cartridge was washed with 10 ml Methanol followed by 10 ml water, then 10 ml of urine sample was passed through cartridge. After passing urine sample 10 ml of 10% Methanol was passed from cartridge so as to remove the weakly bound components. Then 3 ml pure Methanol was passed through cartridge and eluate was collected and filtered with 0.45 µm filter paper and 20 µl from that was injected into HPLC for the analysis.

Results and Discussion:

Urine samples from healthy volunteers were cleaned-up using solid-phase extraction procedure and run as blank. Chromatogram shows the retention of some endogenous compounds which may be urinary free steroid as inferred by adding cortisone to urine sample and comparing the UV spectra (Fig. 1). No further attempt was made to separate or identify endogenous steroid due to non-availability of standards. Keeping in view the retention of endogenous compounds mobile phase was optimized for separation of betamethasone and prednisolone and found to be methanol–water with 44:56, respectively with flow rate of 2.00 ml/min (Fig.2). As the proposed activity was intended to develop a method that can be used in routine analysis, the system was validated systematically. The parameters used for the method validation were, system performance, linearity and calibration, reproducibility and intra-day precision, reproducibility and inter-day precision, analysis of corticosteroids in tablets and urine samples and the robustness of method was validated by checking the slight variations in flow rate, methanol content, injection volume and the wavelength of detection.

The system performance was calculated by the reproducibility tests of the retention time, number of theoretical plates, capacity factor, resolution and the relative retention of corticosteroids (Table 1). The linearity and calibration of corticosteroids was determined in the range of 1 to 10 μg mL-1 with detection limit, calculated by the classical method of 3σ and it was found to be 1.1ng, 0.75ng for the prednisolone and betamethasone respectively (Table 2).

The reproducibility test of the method was determined by running 5 samples of the known concentration daily and for consecutive five days and it was found reproducible in both intra and inter day precision analysis. The coefficient of variance was 1.16 and 1.09 for intra-day analysis and 1.52 and 3.10 for inter-day precision analysis of prednisolone and betamethasone respectively (Table 3, 4).

The robustness of the method was determined by calculating the slight variations in analytical conditions (Table 5). Flow rate of mobile phase did not showed any significant change in the resolution of the peaks but only the variations were noted in the retention times but slight variations of the Methanol content in the mobile phase were determined to be very sensitive for both retention time and resolution for three corticosteroids, so the methanol content in the mobile phase is to be controlled carefully so as to attain the good separation. The amount of sample injected into the HPLC was also determined to be very sensitive because the slight variations in the sample amount injected have significant effect on the %recovery though no change in separation was observed. This shows high capacity of monolith column and could be helpful in enhancing sensitivity by using higher sample volumes. Wavelength was not found much sensitive as all the recoveries are in the range of ± 5%.

Urine samples

The spiked urine samples were analyzed by above mentioned procedure. Fig.3 shows the clean chromatogram with baseline separation from endogenous compounds and two steroids are completely resolved. Percent recovery ranges from 97%-103% for the five samples assayed.

Conclusion and future work

With the proposed method a satisfactory separation of the analytes, extended linear range and rapid analysis time is carried out. The corticosteroids were separated in less than 7 minutes. A good recovery of each steroid was achieved using Monolithic column, which indicate a good agreement with corticosteroid amount spiked in samples. The proposed HPLC method ensures a precise and accurate determination of prednisolone, and betamethasone in urine samples. Work is in progress to resolve other corticosteroids along with prednisolone and betamethasone. Applications will be extended to determine corticosteroids blood and urine samples for screening and diagnostic purposes.

Figure 1 Chromatogram of urine showing both normal and spiked urine sample.

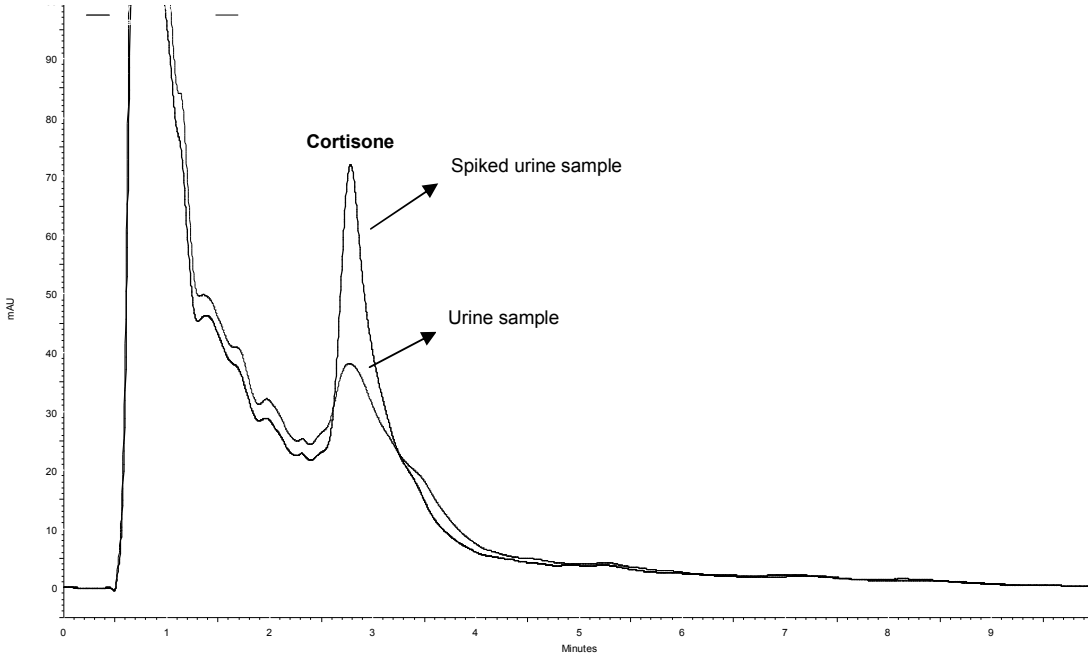

Figure 2 Separation of prednisolone (1) and betamethasone(2) using monolith column, methanol-water (44 : 56) as mobile phase @ 254 nm with flow rate 2.0 ml/min.

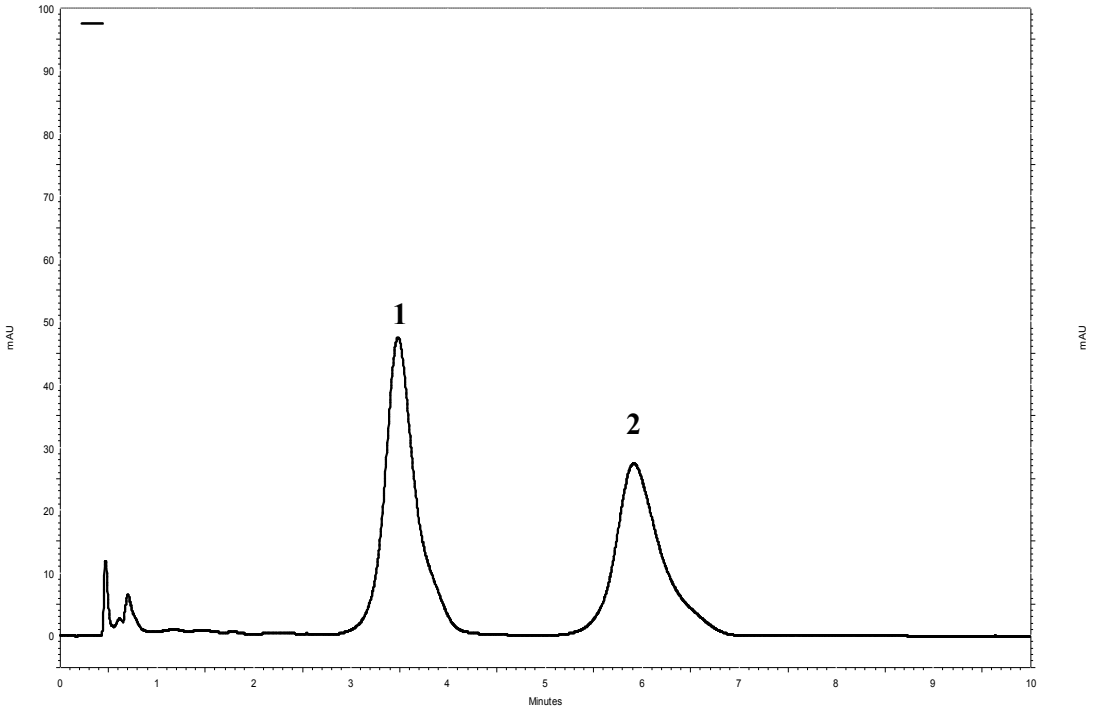

Figure 3 Urine samples spiked with prednisolone and betamethasone after clean up by solid phase extraction

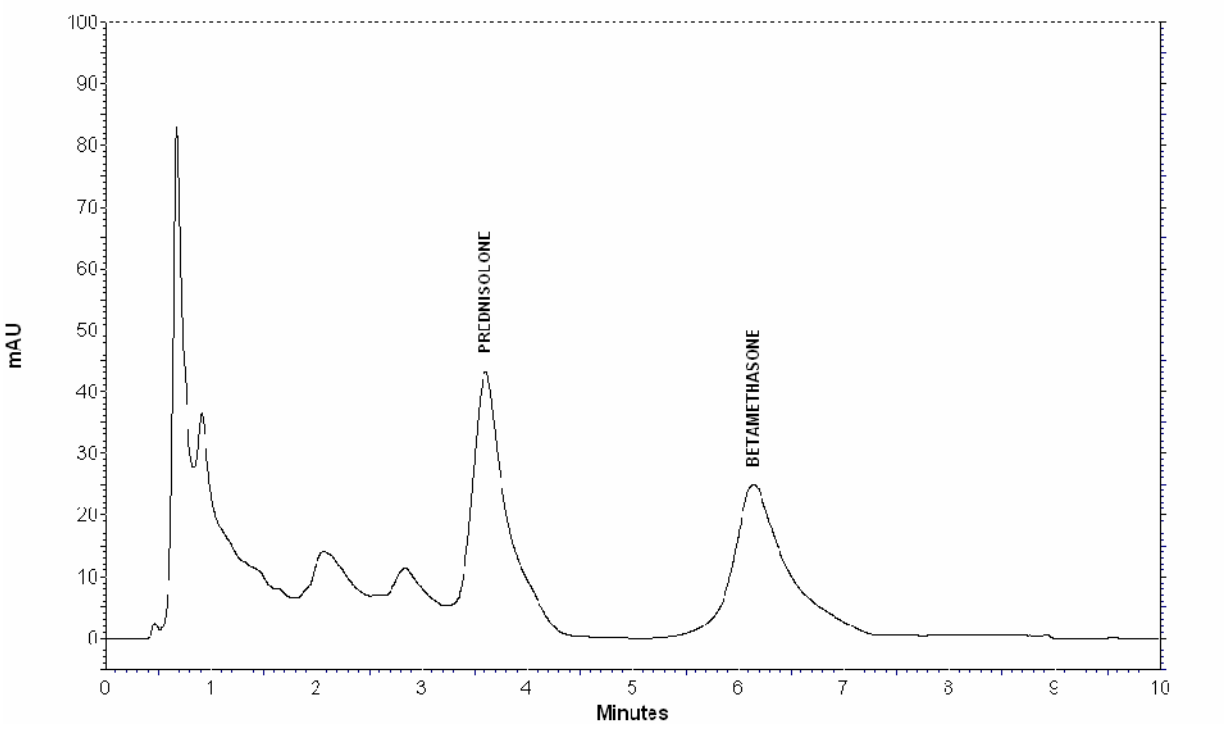

Table 1 System Performance for used corticosteroids (n = 5):

Compound	$t_R \pm SD$ (min)	N	k	R_S	α
Prednisolone	3.47 ± 0.03	1229	3.448	2.594	1.302
Betamethasone	5.77 ± 0.04	2317	6.397	6.852	1.623

Table 2 Linearity and the calibration:

Compound	Detection Limit (ng per injection)	Range of Calibration	Slope	Intercept	Regression coefficient
Prednisolone	1.1 ng	1 To 10 µg mL^{-1}	2.175	0.12	0.9982
Betamethasone	0.75 ng	1 To 10 µg mL^{-1}	1.683	-0.13	0.9982

Table 3 Reproducibility and inter-day precision of steroid derivatives:

Compound	Used concentration µg mL^{-1}	Observed concentration	C.V (%)*	Accuracy (%)**
Prednisolone	4.5	4.69 ± 0.05	1.16	104.24
Betamethasone	4.5	4.59 ± 0.05	1.09	102.21

* Coefficient of Variance (%) = S.D x 100/mean

** Accuracy (%) = observed concentration x 100/used concentration

Table 4 Reproducibility and intra-day precision of steroid derivatives:

Compound	Used concentration µg mL^{-1}	Observed concentration	C.V (%)*	Accuracy (%)**
Prednisolone	4.5	4.67 ± 0.07	1.52	103.86
Betamethasone	4.5	4.67 ± 0.14	3.10	103.83

Table 5 Robustness parameters for corticosteroids

Compound	Flow Rate (ml/min)			Methanol Content			Wavelength (nm)			Sample Amount (µl)		
	1.9	2.0	2.1	43	44	45	252	254	256	16	18	20
	Retention time (min)						% Recovery					
Prednisolone	3.79	3.62	3.43	4.04	3.62	3.35	99.58	102.78	97.15	87.5	96.7	102.5
Betamethasone	6.46	6.16	5.83	7.07	6.16	5.59	104.90	103.21	100.0	88	97.5	103.7

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