Pak. j. sci. ind. res. Ser. A: phy. sci. 2011 **54** (2) 103-107

HPLC Determination of Betamethasone and Prednisolone in Urine Samples Using Monolithic Column

Kamran Abro[ab]*, Najma Memon[a], Muhammad Iqbal Bhanger[a], Shahnaz Perveen[b] and Rehana Jafri[b]

[a]National Centre of Excellence in Analytical Chemistry, University of Sindh, Jamshoro - 76080, Pakistan
[b]PCSIR Laboratories Complex, Shahrah-e-Dr. Salimuzzaman Siddiqui, Karachi - 75280, Pakistan

(received February 24, 2010; revised August 13, 2010; accepted August 18, 2010)

Abstract. A fast and reliable HPLC method is reported for the separation and quantification of betamethasone and prednisolone in urine samples using Chromolith® Performance RP-18e (100 mm × 4.6 mm) column. The separation and detection was achieved using an isocratic mobile phase composed of methanol:water (44:56 v/v) at 2.0 mL/min and wavelength of 254 nm. After successful optimisation of method parameters, it was applied to the urine samples. Solid phase extraction technique was used to clean the sample before analysis. The developed method was validated for the system suitability, precision and accuracy. The limits of detection for the prednisolone and betamethasone are 0.11 ng and 0.075 ng/10 µL injection, respectively allowing their determination in human urine samples. Recovery for spiked urine samples was in the range of 97-103%. The method offers a valuable alternative to the methodologies currently employed for separation and quantification of prednisolone and betamethasone in urine samples.

Keywords: monolithic column, HPLC, betamethasone, prednisolone, urine, solid phase extraction

Introduction

Corticosteroids are a family of drugs which include cortisol (hydrocortisone), an adrenal hormone found naturally in the body, as well as synthetic drugs. Though natural and synthetic corticosteroids, both are 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 (Nozaki, 2001). Assay of steroids is important in pharmaceutical formulations (Hashem and Jira, 2005) and in biological fluids for disease diagnosis (Lin *et al*., 1997), in pharmacokinetics (Glówka *et al*., 2006), in study of metabolism of selected steroids (Kartsova *et al*., 2004; Gallego and Arroyo, 2002) and as a test for doping and veterinary control (Touber *et al*., 2007; Baiocchi *et al*., 2003). Separation techniques like HPLC (Frerichs and Tomatore, 2004), GC (Vanluchene and Vandekerckhove, 1985) and CE (Jumppanen *et al*., 1994) are reported to separate and determine multiple steroids and single analyte of interest from its interfering components. For separation of analytes from endogenous materials, use of coupled column chromatography with mass spectrometric (Polettini *et al*., 1998) or tandem MS have been reported (Tamvakopoulos *et al*., 2002).

Monolith columns are new generation in HPLC stationary phases. Silica-based monoliths have small-size skeletons and a bimodal pore size distribution with µm-sized throughpores and nm-size mesopores, which impart silica-based monoliths favourable properties of high-efficiency, fast separation through low-pressure drop across the column, fast mass transfer kinetics and high binding capacity (Rieux *et al*., 2005). Many successful applications have been reported in pharmaceutical and biological analysis (Satínský *et al*., 2006; Hashem and Jira, 2005; Zarghi *et al*., 2005). Monolithics have found applications in assay of corticosteroids as well (Valencia *et al*., 2008; 2007).

Here, monolithic column was used for separation of betamethasone – a corticosteroid known for its effects on nervous system, carbohydrate metabolism and cardiovascular system (attractive for drug doping) – from predinisolone and urinary endogenous compounds (Polettini *et al*., 1998).

Materials 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

*Author for correspondence; E-mail: abrokamran@gmail.com

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).

Separation was achieved with the analytical column Chromolith® Performance RP-18e (100 mm × 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. Detection was achieved at 254 nm. ChromQuest software was used for data analysis. Solid-phase extraction was carried out using Supelco Discovery DSC-18 (PA, USA) SPE cartridges.

Standard solutions of the corticosteroids. Stock solutions of 1000 µg/mL (1 mg/mL) of each corticosteroid were prepared in methanol. Working standard solutions were prepared by diluting aliquots of each stock solution to obtain concentrations ranging from 1 to 10 µg/mL. Calibration graphs were constructed by plotting the peak areas obtained at wavelength 254 nm *vs* the corresponding injected amounts (ng).

Urine sample preparation. Urine samples were collected from five volunteers. Steroids were determined by spiking urine samples of healthy persons to get final concentration of 5 µg/mL and extracted using solid phase extraction technique. SPE cartridge was washed with 10 mL methanol followed by 10 mL water, then 10 mL of urine sample was passed through the cartridge. After that, 10 mL of 10% methanol was passed through the cartridge to remove weakly bound components. Then 3 mL of methanol was passed through the cartridge at the flow rate of 3 mL/min. Finally, eluate was collected and filtered with 0.45 µm filter paper and then 10 µL of the filtrate 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 steroids due to non-availability of standards. Keeping in view the retention of endogenous compounds, mobile phase was modified to achieve the separation of betamethasone and prednisolone and found to be methanol-water 44:56 (v:v), respectively, with a 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 being system performance, linearity and calibration, reproducibility and intra-day precision. Analysis of corticosteroids in tablets and urine samples and the robustness of method were 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 with detection limit,

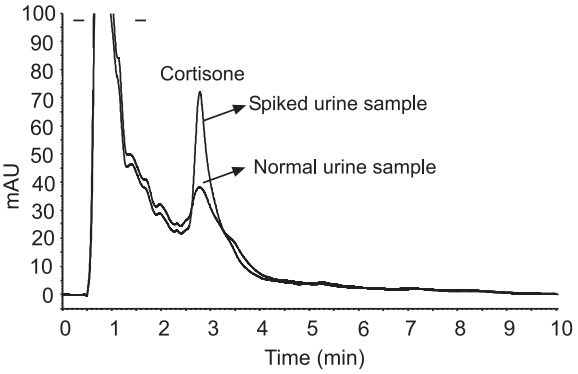

Fig. 1. Chromatogram of urine showing both normal and spiked urine samples.

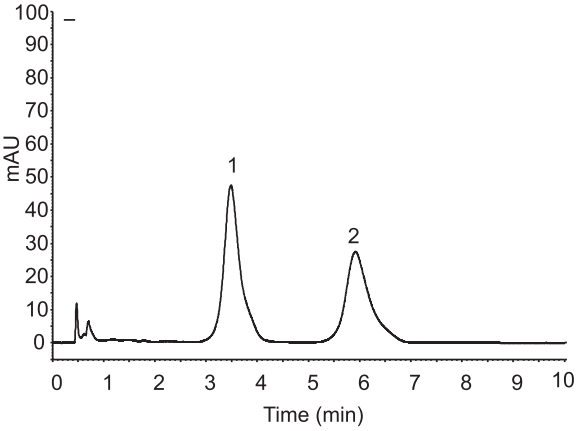

Fig. 2. Separation of prednisolone 5 µg/mL and betamethasone 5 µg/mL, using monolithic column methanol-water (44:56) as mobile phase @ 254 nm with flow rate 2.0 mL/min.

calculated by the classical method of 3σ and it was found to be 1.1 ng and 0.75 ng with equation of straight line, y = 2.175 χ + 0.12 and y = 1.683 χ - 0.13, for the prednisolone and betamethasone, respectively. The reproducibility test of the method was determined by running 5 samples of known concentration daily and for consecutive five days and it was found reproducible in both intra-and inter-day analysis precision analysis. The coefficient of variance was 1.16 and 1.09 for intra-day and 1.52 and 3.10 for inter-day precision of prednisolone and beta-methasone, respectively (Table 2).

The robustness of the method was determined by calculating slight variations in analytical conditions (Table 3). Flow rate of mobile phase did not show any significant change in the resolution of the peaks. Only variations were noted in the retention time but slight variations of methanol content in the mobile phase were determined to be very sensitive for both retention time and resolution for two corticosteroids, so the methanol content in the mobile phase is to be controlled carefully to attain the separation. The amount of sample injected into the HPLC was also determined to be very sensitive because slight variation in the sample amount injected had significant effect on the percent recovery though no change in the 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%.

Table 1. System performance for corticosteroids (n = 5)

Compound	^{t}R ± SD (min)	N	k	RS	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. Reproducibility, inter-day and intra-day precision of predinisolone and betamethasone

Compound	Inter-day analysis			
	Used concentration (µg/mL)	Observed concentration (µg/mL)	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
		Intra-day analysis		
Prednisolone	4.5	4.67 ± 0.07	1.52	103.86
Betamethasone	4.5	4.67 ± 0.14	3.10	103.83

* = coefficient of variance (%) = SD × 100/mean; ** = accuracy (%) = observed concentration × 100/used concentration.

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

Conclusion and future work. With the proposed method, satisfactory separation of the analytes, extended linear range and rapid analysis time was achieved. The

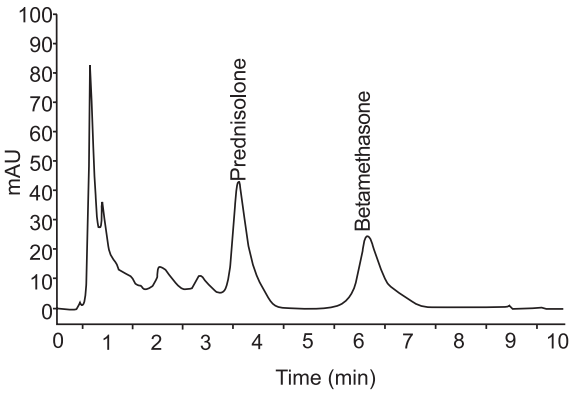

Fig. 3. Urine samples spiked with prednisolone and betamethasone after clean up by solid phase extraction.

Table 3. 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	87.50	96.70	102.50
Betamethasone	6.46	6.16	5.83	7.07	6.16	5.59	104.90	103.21	100.00	88.00	97.50	103.70

corticosteroids were separated in less than 7 min. Good recovery of each steroid was achieved using monolithic column, which indicated good agreement with corticosteroid amount spiked in samples. The proposed HPLC method ensures precise and accurate determination of prednisolone and betamethasone in urine samples. Work is in progress to resolve other corticosteroids along with prednisolone and betamethasone. Application will be extended to determine corticosteroids in blood samples for screening and diagnostic purposes.

References

Baiocchi, C., Brussino, M., Pazzi, M., Medana, C., Marini, C., Genta, E. 2003. Separation and determination of synthetic corticosteroids in bovine liver by LC-Ion-Trap-MS-MS on porous graphite. *Chromatographia*, **58:** 11-14.

Frerichs, V.A., Tomatore, K.M. 2004. Determination of the glucocorticoids prednisone, prednisolone, dexamethasone and cortisol in human serum using liquid chromatography coupled to tandem mass spectrometry. *Journal of Chromatography B*, **802:** 329-338.

Gallego, J.M.L., Arroyo, J.P. 2002. Simultaneous determination of dexamethasone and trimethoprim by liquid chromato-graphy. *Journal of Pharmaceutical and Biomedical Analysis*, **30:** 1255-1261.

Glówka, F.K., Karazniewicz, M., Lipnicka, E. 2006. RP-HPLC method with fluorescence detection for determination of small quantities of triamcinolone in plasma in presence of endogenous steroids after derivatization with 9-anthroyl nitrile; pharmacokinetic studies. *Journal of Chromatography B*, **839:** 54-61.

Hashem, H., Jira, T. 2005. Chromatographic applications on monolithic columns: Determination of triamcinolone, prednisolone and dexamethasone in pharmaceutical tablet formulations using solid phase extraction and monolithic column. *Chromatographia*, **61:** 133-136.

Jumppanen, J.H., Wiedmer, S.K., Siren, H., Riekkola, M.L., Haario, H. 1994. Optimized separation of seven cortico-steroids by micellar electrokinetic chromatography. *Electrophoresis*, **15:** 1267-1272.

Kartsova, A.A., Velikanova, L.I., Pavlova, E.G., Bessonova, E.A. 2004. Steroidogenesis in patients with various adrenal cortex diseases as studied by reversed-phase high-performance liquid chromatography. *Journal of Analytical Chemistry*, **59:** 976-982.

Lin, C.L., Wu, T.J., Machacek, D.A., Jiang, N.S., Kao, P.C. 1997. Urinary free cortisol and cortisone determined by high performance liquid chromatography in the diagnosis of Cushing's syndrome. *Journal of Clinical Endocrinology Metabolism*, **82:** 151-155.

Nozaki, O. 2001. Steroid analysis for medical diagnosis. *Journal of Chromatography A*, **935:** 267-278.

Polettini, A., Bouland, G.M., Montagna, M. 1998. Development of a coupled-column liquid chromatographic-tandem mass spectrometric method for the direct determination of betamethasone in urine. *Journal of Chromatography B: Biomedical Sciences and Applications*, **713:** 339-352.

Rieux, L., Niederländer, H., Verpoorte, E., Bischoff, R. 2005. Silica monolithic columns: Synthesis, characterisation and applications to the analysis of biological molecules. *Journal of Separation Science*, **28:** 1628-1641.

Satínský, D., Huclová, J., Ferreira, R.L., Montenegro, M.C., Solich, P. 2006. Determination of ambroxol hydrochloride, methylparaben and benzoic acid in pharmaceutical preparations based on sequential injection technique coupled with monolithic column. *Journal of Pharmaceutical and Biomedical Analysis*, **40:** 287-293.

Tamvakopoulos, C.S., Neugebauer, J.M., Donnelly, M., Griffin, P.R. 2002. Analysis of betamethasone in rat plasma using automated solid-phase extraction coupled with liquid chromatography-tandem mass spectrometry. Determination of plasma concentrations in rat following oral and intravenous administration. *Journal of Chromatography B*, **776:** 161-168.

Touber, M.E., van Engelen, M.C., Georgakopoulus, C., van Rhijin, J.A., Nielen, M.W.F. 2007. Multidetection of corticosteroids in sports doping and veterinary control using high-resolution liquid chromatography/time-of-flight mass spectrometry. *Analytica Chimica Acta*, **586:** 137-146.

Valencia, R.M., Lumbreras, R.G., Montes, A.S., Hornillos, R.I. 2008. Quantitative screening for steroids in animal feeding water using reversed phase LC with gradient elution. *Journal of Separation Science*, **31:** 219-228.

Valencia, R.M., Lumbreras, R.G., Montes, A.S., Hornillos, R.I. 2007. Method development validation for corticoids in animal feed samples

by liquid chromatography using a monolithic column. *Journal of Separation Science*, **30:** 2950-2957.

Vanluchene, E., Vandekerckhove, D. 1985. Total steroid profiling with capillary GC. Details on sample preparation. *Journal of High Resolution Chromatography*, **8:** 400-403.

Zarghi, A., Shafaati, A., Foroutan, S.M., Khoddam, A. 2005. Development of a rapid HPLC method for determination of famotidine in human plasma using a monolithic column. *Journal of Pharmaceutical and Biomedical Analysis*, **39:** 677-680.