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Simple liquid chromatography method for the rapid simultaneous determination of prednisolone and cortisol in plasma and urine using hydrophilic lipophilic balanced solid phase extraction cartridges

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Abstract

This article describes the development and validation of a simple solid phase extraction (SPE) and HPLC method for the extraction and the specific determination of prednisolone and hydrocortisone (cortisol) in both plasma and urine using one washing step with Oasis® hydrophilic lipophilic balanced (HLB) cartridges (1 ml/30 mg, 30 μm). Recoveries of prednisolone and cortisol from plasma and urine exceeded 82%. The limit of quantification (LOQ) in plasma and urine was 9.9 and 6.7 ng/ml for cortisol, respectively, and 11.6 and 8.0 ng/ml for prednisolone, respectively. The intraday and interday precision (measured by CV%) for both prednisolone and cortisol in both plasma and urine was always less than 7%. The accuracy (measured by relative error %) for both prednisolone and cortisol in both plasma and urine was always less than 8%. The advantages of the developed method are the use of a one step washing SPE utilising HLB cartridges which do not suffer the drying out problems of conventional SPE cartridges and the time saving when compared with solvent extraction (SE), in addition to the simultaneous determination of prednisolone and cortisol in both plasma and urine.

Keywords: Steroids; Prednisolone; Cortisol

1. Introduction

Prednisolone is used extensively as an anti-inflammatory and immunosuppressive agent [1]. One of the main side effects of prednisolone and inhaled steroids is the suppression of plasma and urinary cortisol (hydrocortisone) [2]. The most common method for determining cortisol suppression is measuring the 24 h urinary excretion together with the morning plasma cortisol level. The measurement of plasma levels of prednisolone can be of benefit to clinicians to exclude the risk of subtherapeutic concentrations, e.g. in the case of non-compliance with prescribed therapy or in assessing overdose.

Simultaneous determination of prednisolone and endogenous cortisol in both plasma and urine can therefore be useful for monitoring prednisolone therapy. Monitoring cortisol suppression is also important for patients on high dose inhaled steroids. By conducting a computer literature search (Medline 1966–2002) more than 50 articles have been cited concerning the determination of prednisolone in plasma or urine or both and more than 200 articles were concerned with the determination of cortisol in plasma or urine or both. Only three articles, however, were cited detailing the determination of both cortisol and prednisolone in both plasma (or serum) and urine [3–5]. A serious problem with most of these methods is the lack of specificity in the determination of urinary cortisol [6]. Many methods use radio immuno assay (RIA) [7,8] which has the disadvantage of interference with antibody analyte binding [9]. Other methods use HPLC–mass spectrometry [10–12] which has the disadvantage of being expensive and not available in all laboratories. Some of these methods use fluorimetric derivatisation before HPLC analysis [5,13] which is limited by the precisely controlled reaction conditions and the instability of the fluorescent analogue [9]. Many of these methods give rise to long retention times for prednisolone and cortisol of up to 20 min [4,14,15]. Many of the methods use solvent extraction (SE) in sample preparation [4,14,15] which is

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time consuming and has many disadvantages [16,17]. Some methods use solid phase extraction (SPE) for sample preparation, however, they use conventional silica based cartridges which can lead to problems due to the cartridges running dry during the extraction, which in turn can cause variable recovery [18]. Most of the methods are only validated for plasma [12,14,15,19–21] or urine analysis [7,17,22] but not both.

This paper describes the development and validation of a solid phase extraction/HPLC/UV method utilising hydrophilic lipophilic balanced (HLB) SPE cartridges for the simultaneous determination of prednisolone and cortisol in human plasma and urine. The validation of the method and the quality assurance during routine analysis was carried out according to standard protocols.

2. Experimental

2.1. Reagents

Prednisolone, beclomethasone, and cortisol were purchased from Sigma (Poole, England) and were of a minimum purity of 99%. Methanol, dichloromethane (DCM) and tetrahydrofuran (THF) were of HPLC grade. Acetic acid and diethyl ether were of analytical reagent grade. All solvents were purchased from Romil (Cambridge, UK). Membrane filters F-450 0.45 μm were obtained from Gelman Laboratory (Portsmouth, UK).

2.2. Instrumentation

Solid phase extraction cartridges (Oasis® HLB (1 ml, 30 mg) were purchased from Waters (AGB, Belfast). Extraction was carried out using a Waters extraction manifold. The chromatographic system consisted of a Shimadzu LC-GA pump, Waters 712 WISP autosampler and UV absorbance detector (LDC 12 Milton Roy, Riviera Beach, USA). The separation was performed using a Thermo Hypersil silica analytical column (250 mm × 4.6 mm, 5 μm; Thermo Hypersil Ltd., Runcorn, UK). The guard column was a Thermo Hypersil silica (20 mm × 4 mm, 5 μm; Thermo Hypersil Ltd., Runcorn, UK). Data recording was carried out using Shimadzu Class VP software.

2.3. Preparation of cortisol free plasma and cortisol free urine

The blood was donated from the Northern Ireland Blood Transfusion Centre. The urine was obtained from volunteers. Four grams of activated charcoal were added to 100 ml plasma or urine and mixed using a magnetic stirrer for 2 h. The mixture was then centrifuged for 3 h at 3000 rpm (1610 × *g*). After centrifugation the supernatant plasma or urine was filtered using a sintered glass filter (grade 4) [4,14].

2.4. Internal standard

Beclomethasone was used as the internal standard. The concentration of the internal standard for plasma was 150 ng/ml for plasma and 100 ng/ml of urine.

2.5. Standards

To 1 ml of cortisol free plasma, 50 μl of methanolic solutions of prednisolone and cortisol and 50 μl of methanolic solution of the internal standard were added resulting in the following plasma calibration standards: 25, 50, 100, 200, 400, 600, and 800 ng/ml plasma and 150 ng/ml plasma for the internal standard. For urine a similar procedure was used resulting in the following urine calibration standards: 10, 25, 50, 75,100, 150, and 200 ng/ml urine and 100 ng/ml urine for the internal standard.

2.6. Sample handling and preparation of plasma and urine samples from patients

Patients' blood samples were collected into glass tubes containing EDTA and centrifuged at 3000 rpm (1610 × *g*) for 15 min and the plasma separated. The plasma was kept frozen at −70 °C until analysis. To 1 ml of the individual plasma samples 50 μl of methanol and 50 μl of a methanolic solution of the internal standard (150 ng/50 μl) were added. Urine samples were stored immediately after collection at −70 °C until analysis. To 1 ml of the individual urine samples 50 μl of methanol and 50 μl of a methanolic solution of the internal standard (100 ng/50 μl) were added.

2.7. Extraction procedure

The samples were prepared as detailed above and then extracted using Oasis® HLB cartridges (1 ml, 30 mg) with a Waters' extraction vacuum manifold as follows:

1. *Condition*: 1 ml methanol followed by 1 ml of water.
2. *Load*: 1.1 ml spiked plasma (or urine) (as prepared above).
3. *Wash*: 1 ml 2% NH_4OH in 40% methanol for plasma samples and 2% NH_4OH in 50% methanol for urine samples.
4. *Elute*: 1 ml methanol.
5. Evaporate the eluate with nitrogen stream, reconstitute in 350 μl of the mobile phase and inject 150 μl onto the HPLC column.

In order to determine the best washing solvent to be used at step three, different percentages of methanol were tested (10, 15, 20, 25, 30, 40, 50, 60, 70, and 80%) and then the recovery calculated.

2.8. Chromatography

The mobile phase was pumped at a flow rate of 2 ml/min and consisted of dichloromethane (66.45% (v/v)); water

saturated dichloromethane (30% (v/v)), methanol (2.5 % (v/v)), tetrahydrofuran (1% (v/v)) and glacial acetic acid (0.05% (v/v)). Detection was by UV absorption at 240 nm. The prepared mobile phase was filtered through a 0.45 μm Millipore filter and degassed ultrasonically before use.

2.9. Assay characteristics for method validation

2.9.1. Specificity

To demonstrate the specificity of the method, blank plasma and urine samples and plasma and urine samples from patients who had been prescribed prednisolone were used. Representative chromatograms were generated to show that the extraneous peaks are resolved from the peaks for prednisolone and cortisol. In order to further check the purity of the cortisol peak during the analysis of urine samples, the absorbance ratio for cortisol in patients' urine samples ($n = 4$) at two different wavelengths (240 and 255 nm) was measured and compared with the ratio obtained from spiked mobile phase (50 ng/ml).

2.9.2. Standard curves and linearity

Standard curves (cortisol and prednisolone) were determined on each day of a 5-day validation; the slopes, the intercepts and the correlation coefficients were determined. For calculation of the standard curve characteristics plots of peak height ratios against concentration were used.

2.9.3. Accuracy and precision

Intraday precision, interday precision and the accuracy were calculated from data obtained during a 5-day validation. Three concentrations were chosen from the high medium and low range of the standard curve (50, 200, and 600 ng/ml for plasma and 25, 75, and 150 ng/ml for urine) for both prednisolone and cortisol. Plasma and urine samples spiked at these three concentrations were analysed on each day of the 5-day validation ($n = 5$ at each concentration). Precision was expressed as the coefficient of variation (CV%). Accuracy was expressed as the mean relative error (RE%). A precision (CV%) $\leq$15% and an accuracy (RE%) $\leq$15% are acceptable [15].

2.9.4. Limit of quantification (LOQ) and limit of detection (LOD)

Ten independent blank samples were measured once each. The LOD was expressed as the analyte concentration corresponding to the sample blank value plus 3 standard deviations. Limit of quantification (LOQ) was expressed as the analyte concentration corresponding to the sample blank value plus five standard deviations [23].

2.9.5. Recovery

The recovery was calculated according to the following formula: recovery = (peak height for extracted analyte/peak height for solution of analyte) × 100%. The recoveries of prednisolone and cortisol from plasma and urine were determined at three concentrations 50, 200, and 600 ng/ml for plasma and 25, 75 and 150 ng/ml for urine, the recovery of the internal standard was determined at the concentration used which is 100 and 150 ng/ml.

2.9.6. Stability

Six urine and plasma samples from six different patients were analysed twice at an interval of 3 months to investigate the stability of cortisol after storing the samples at −70 °C. The mean and standard deviation for the ratio between the two measurements were determined. Three plasma samples from three different patients taking prednisolone were analysed twice with an interval of 3 months to check the stability of prednisolone after storing the samples at −70 °C; the mean and standard deviation for the ratio between the two measurements were again determined.

2.10. Calculations

Standard regression curve analysis was computed using Shimadzu Class VP software without forcing through zero. Means and standard deviations were calculated using EXCEL® software (Microsoft Corporation, USA).

3. Results and discussion

3.1. Optimisation of the extraction techniques

In the original method reported by Delargy [24] prednisolone and cortisol were extracted from plasma using solvent extraction. Many problems were found with this including emulsion formation, loss of sample and the extraction was time consuming, as each sample needs to be extracted separately. The overall process of standard preparation, extraction and running took approximately 10 h (just for calibration curve preparation). It was obvious that another method of extraction should be used; searching the literature indicated that solid phase extraction (SPE) could be used for the extraction of steroids from plasma and urine [9]. Recently polymer based SPE cartridges have been introduced (e.g. Oasis® HLB). HLB is an acronym for hydrophilic–lipophilic balance which describes the two key features of the polymer: the ability to remain wetted and the ability to adsorb or retain analytes, the use of these cartridges permits a more precise and less tedious process than with the conventional silica based SPE allowing simultaneous analysis of a higher number of samples [18].

The general Oasis® HLB SPE procedure is as follows:

1. *Condition*: 1 ml methanol followed by 1 ml of water.
2. *Load*: 1 ml spiked plasma.
3. *Wash*: 1 ml 5% methanol in water.
4. *Elute*: 1 ml methanol.
5. Evaporate with nitrogen stream and reconstitute in the mobile phase and inject in the HPLC.

In the third step of the extraction procedure (washing step), 5% methanol is suggested by the manufacturer as the washing solvent; the percentage of methanol can be changed in order to increase the efficacy of the cleaning procedure, remove the interferences and increase the recovery.

From the investigation of charcoal stripped plasma samples in this study it was found that 10% methanol washing gave the best recovery. However, when the patients' samples were washed with 10% methanol, a number of endogenous interferences appeared in the chromatograms. By conducting a series of experiments it was found that 2% ammonium hydroxide in 40% methanol was very effective in removing the interferences (Fig. 2). The 2% ammonium hydroxide increased the elution of acidic interferences during the washing step and increased the retention of basic interferences during elution. It was hypothesised that the retention

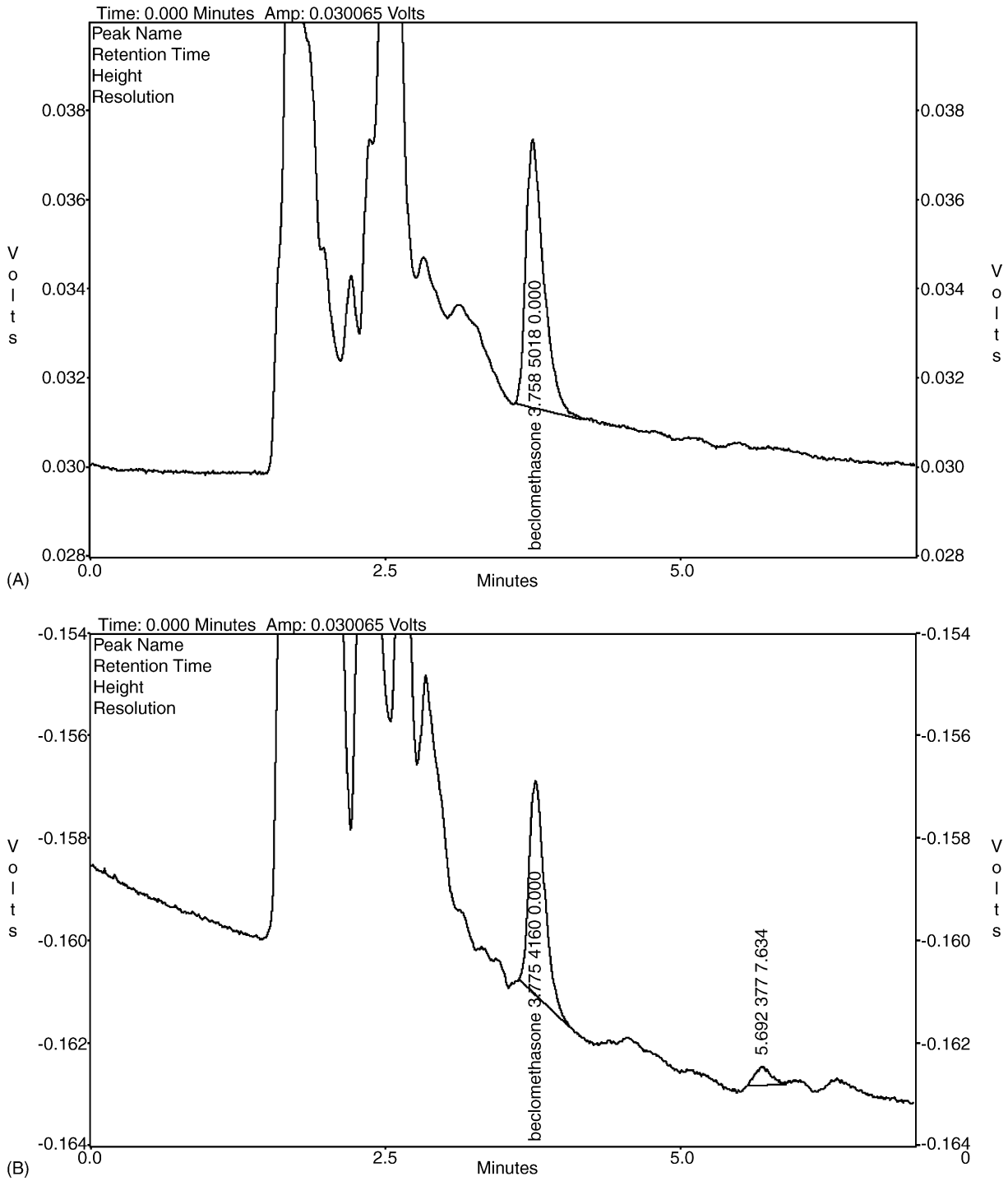

Fig. 1. Chromatograms of extracted plasma (A) and extracted urine (B) taking from asthmatic patients who were on high dose inhaled steroids (2000 μg fluticasone daily) showing no interferences at the retention times of prednisolone or cortisol.

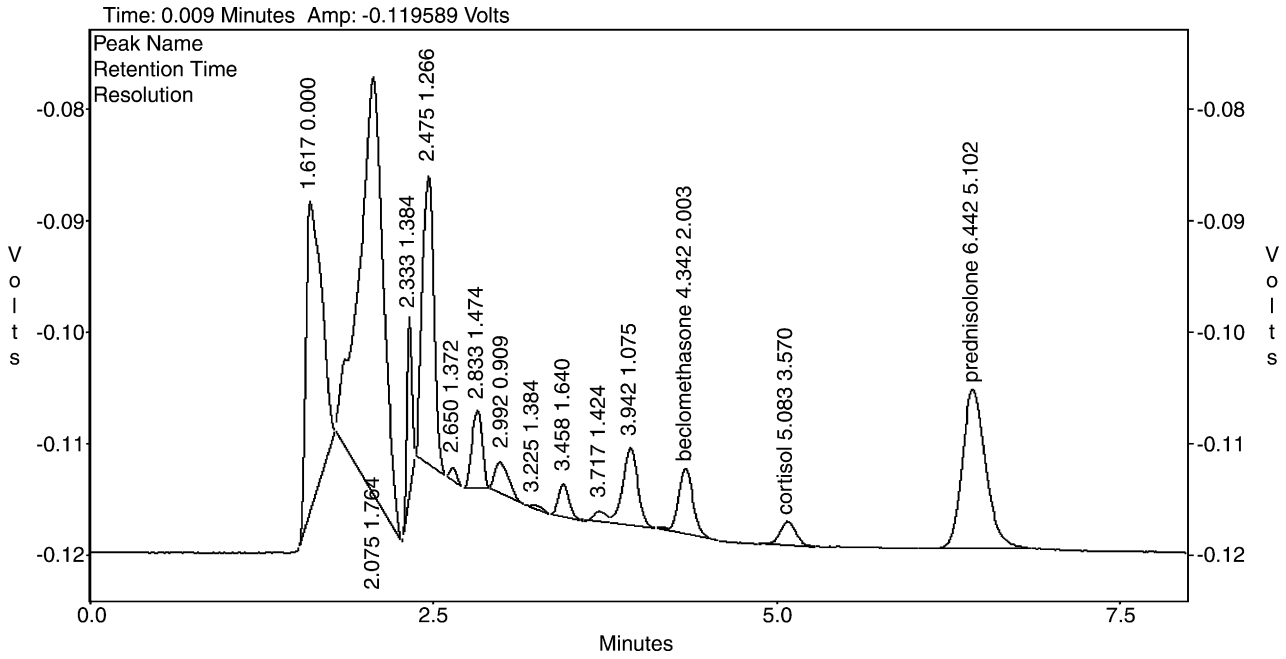

Fig. 2. A chromatogram of extracted plasma sample taken from a patient who was taking 20 mg prednisolone daily. Prednisolone (350.7 ng/ml), cortisol (32.6 ng/ml) (endogenous) and beclomethasone (150 ng/ml) (internal standard) are shown.

of steroids would not be affected, as they are relatively neutral.

The task for urine was more difficult due to the many interferences especially with cortisol. When a 10% methanol washing step was used with unstripped urine samples it was not effective in removing all the interferences. The percentage of methanol needed to be increased so that better washing could be achieved. By conducting a series of experiments it was found that 2% ammonium hydroxide in 50% methanol was very effective in removing the interferences (Fig. 3). Addition of ammonium hydroxide was very effective and significantly improved the clean up of urine samples as compared with using 50% methanol alone (colourless extract obtained as compared to the yellow extract with 50% methanol alone).

It was observed that in some urine samples, despite the clean up procedure, interference was still present. This was solved by an additional washing step with 10% methanol before washing with 50% methanol, without affecting the recovery.

3.2. Validation

3.2.1. Specificity

A chromatogram of extracted plasma (up) and extracted urine (down) taking from asthmatic patients who were on high dose inhaled steroids (2000 μg fluticasone daily) (Fig. 1). The chronic high dose inhaled steroids suppress the endogenous cortisol to levels approximate to zero thereby allowing seeing if there is any interference at the retention time of cortisol. The chromatograms show no interferences at the retention times of prednisolone or cortisol.

Figs. 2 and 3 show chromatograms of extracted plasma and urine sample from a patient who was taking prednisolone. Delargy [24] has also shown that prednisone can be assayed by the mobile phase with acceptable resolution form cortisol and prednisolone.

The absorbance ratio for cortisol in spiked mobile phase was calculated to be 48.8 ± 1.23 and in urine samples was 51.2 ± 1.17. This confirms the suitability of the method for measuring morning urinary cortisol taking into account that a short retention time window must be used (<0.1 min).

3.2.2. Standard curve and linearity

The standard curve was determined on each day of the 5-day validation, the slope, the intercept and the correlation coefficient were determined. Table 1 shows the mean ± S.D.

Table 1
Results of the five calibration curves for prednisolone and cortisol in plasma and urine: slope (mean ± S.D.), intercept (mean ± S.D.) and coefficient of correlation of the standard curves ($n = 2$ at each concentration)[a]

	Slope (mean ± S.D.) (ng/ml)	Intercept (mean ± S.D.)	Correlation coefficient, r
Plasma			
Prednisolone	1.119 ± 0.026	0.101 ± 0.032	>0.997
Cortisol	0.843 ± 0.014	0.105 ± 0.062	>0.998
Urine			
Prednisolone	1.374 ± 0.088	−0.020 ± 0.034	>0.998
Cortisol	0.925 ± 0.020	−0.020 ± 0.035	>0.998

[a] Slopes and intercepts were determined automatically by the Shimadzu Class VP software program for concentration ratio (*Y*-axes) against height ratio (*X*-axes).

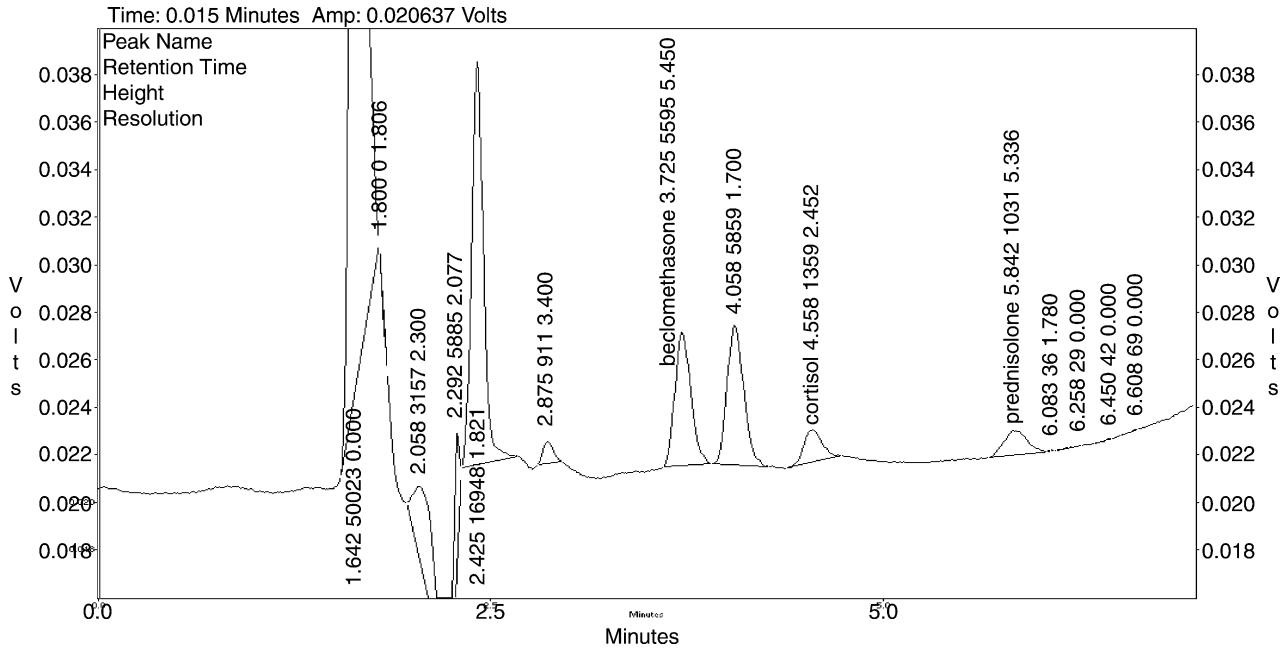

Fig. 3. A chromatogram of extracted urine sample taken from patient who was taking 5 mg prednisolone daily. Prednisolone (23.3 ng/ml), cortisol (20.5 ng/ml) (endogenous) and beclomethasone (100 ng/ml) (internal standard) are shown.

for the slopes and intercepts for five calibration curves for prednisolone and cortisol in plasma and urine. By examining the curves and the table it can be seen that the relationship between peak height ratio and concentration was linear within the studied concentration range.

3.2.3. Accuracy and precision

The values obtained during the 5-day validation for plasma, i.e. intraday and interday precision and accuracy are summarised in Table 2. Table 3 shows the results for the corresponding urine analysis. All values of accuracy and precision were within recommended limits.

3.2.4. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and the LOQ for prednisolone in plasma were calculated to be 7.0 and 11.6 ng/ml, respectively (CV% = 8.0% and mean relative error of 14.3%), and for cortisol 5.9 and 9.9 ng/ml, respectively (CV% = 9.0% and mean relative error of 13.0%) (Fig. 4). The LOD and the LOQ for prednisolone in urine were calculated to be 4.8 and 8.0 ng/ml, respectively (CV% = 8.0% and mean relative error of 14.2%), and for cortisol 4.0 and 6.7 ng/ml, respectively (CV% = 7.5% and mean relative error of 11.0%) (Fig. 5).

3.2.5. Recovery

Table 4 shows the results for extraction recovery from plasma and urine. The recovery of cortisol from plasma ranged between 87.0 and 93.1% and from urine it was between 85.4 and 101.3%. The recovery of prednisolone from plasma ranged between 82.2 and 89.8% and from urine it was between 82.0 and 102.2%.

Table 2
Intraday and interday precision and accuracy (plasma, $n = 5$)

Nominal concentration (ng/ml)	Precision				Accuracy (mean relative errors (%))	
	Cortisol		Prednisolone		Cortisol	Prednisolone
	Mean ± S.D.	CV%	Mean ± S.D.	CV%		
Intraday						
50	52.4 ± 2.7	5.3	49.4 ± 1.9	3.9	4.0	−2.0
200	201.8 ± 1.7	0.9	196.5 ± 1.0	0.5	1.0	−2.0
600	605.3 ± 4.4	0.7	607.9 ± 5.4	0.9	1.0	1.0
Interday						
50	45.7 ± 3.2	7.1	48.5 ± 3.6	7.3	−8.0	−2.0
200	195.3 ± 6.1	3.1	197.9 ± 3.8	1.9	−3.0	−1.0
600	601.2 ± 6.3	1.0	600.1 ± 6.7	1.1	0.1	0.1

Table 3
Intraday and interday precision and accuracy (urine, $n = 5$)

Nominal concentration(ng/ml)	Precision				Accuracy (mean relative errors (%))	
	Cortisol		Prednisolone		Cortisol	Prednisolone
	Mean ± S.D.	CV%	Mean ± S.D.	CV%		
Intraday						
25	25.2 ± 1.7	6.9	25.4 ± 0.8	3.0	0.8	1.6
75	77.5 ± 1.1	1.5	74.2 ± 1.7	2.2	3.3	−1.1
150	149.9 ± 2.3	1.6	144.5 ± 2.5	1.7	0.0	−3.7
Interday						
25	24.6 ± 1.8	7.2	24.0 ± 1.7	7.2	−1.6	−4.0
75	76.2 ± 1.8	2.4	78.0 ± 3.4	4.4	1.6	4.0
150	150.5 ± 2.0	1.3	149.5 ± 1.0	0.7	0.3	−0.3

Table 4
The results of the recovery for P, B and H from plasma and urine ($n = 6$)

Nominal concentration (ng/ml)	Recovery (%)					
	Cortisol		Prednisolone		Beclomethasone	
	Mean ± S.D.	CV%	Mean ± S.D.	CV%	Mean ± S.D.	CV%
Plasma						
50	93.1 ± 5.7	6.1	82.2 ± 5.1	6.1		
200	87.0 ± 6.8	7.8	89.8 ± 5.3	5.9		
600	87.3 ± 11.5	13.1	87.5 ± 11.5	13.5		
150					88.8 ± 4.7	4.7
Urine						
25	101.3 ± 6.2	6.2	102.2 ± 1.4	7.5		
75	90.0 ± 4.5	5.0	84.7 ± 3.0	3.5		
150	85.4 ± 5.6	6.6	82.0 ± 4.6	5.6		
100					83.6 ± 6.2	7.4

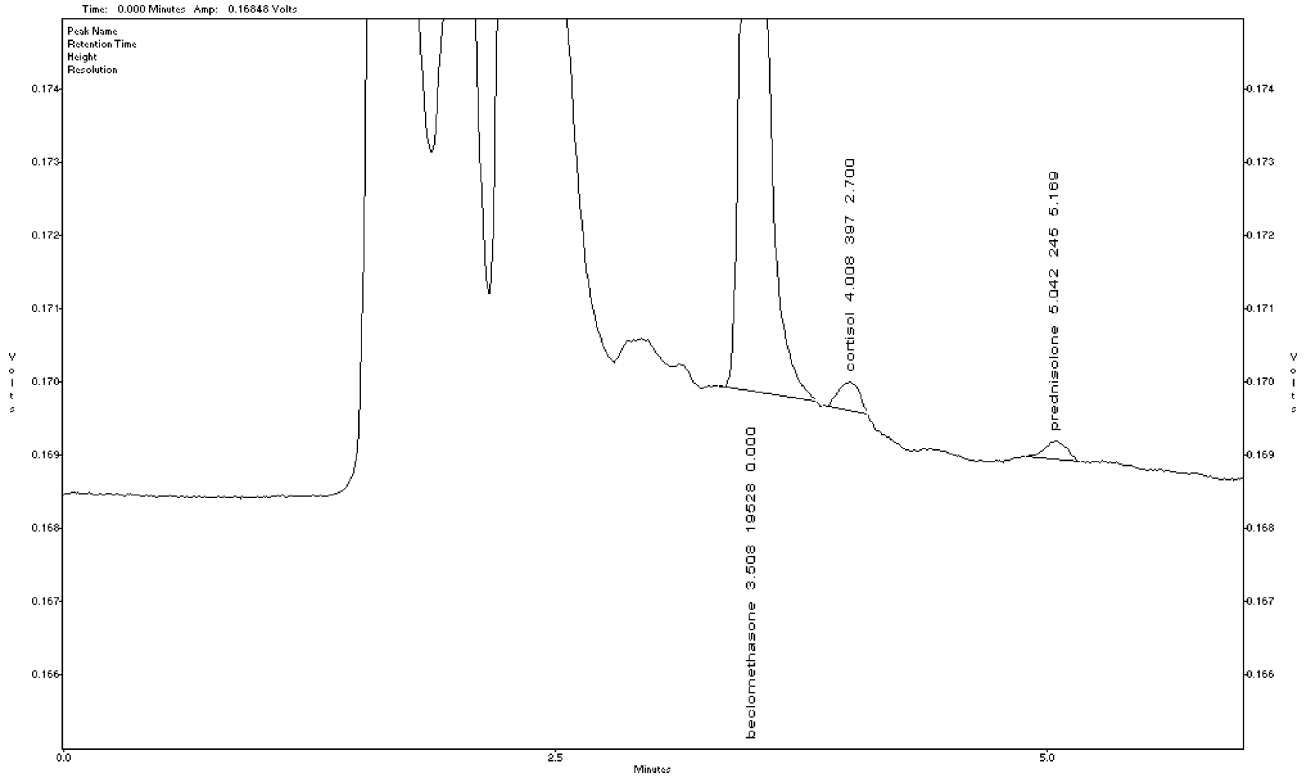

Fig. 4. A chromatogram of extracted plasma spiked at 12 ng/ml prednisolone 10 ng/ml cortisol and 1000 ng/ml beclomethasone.

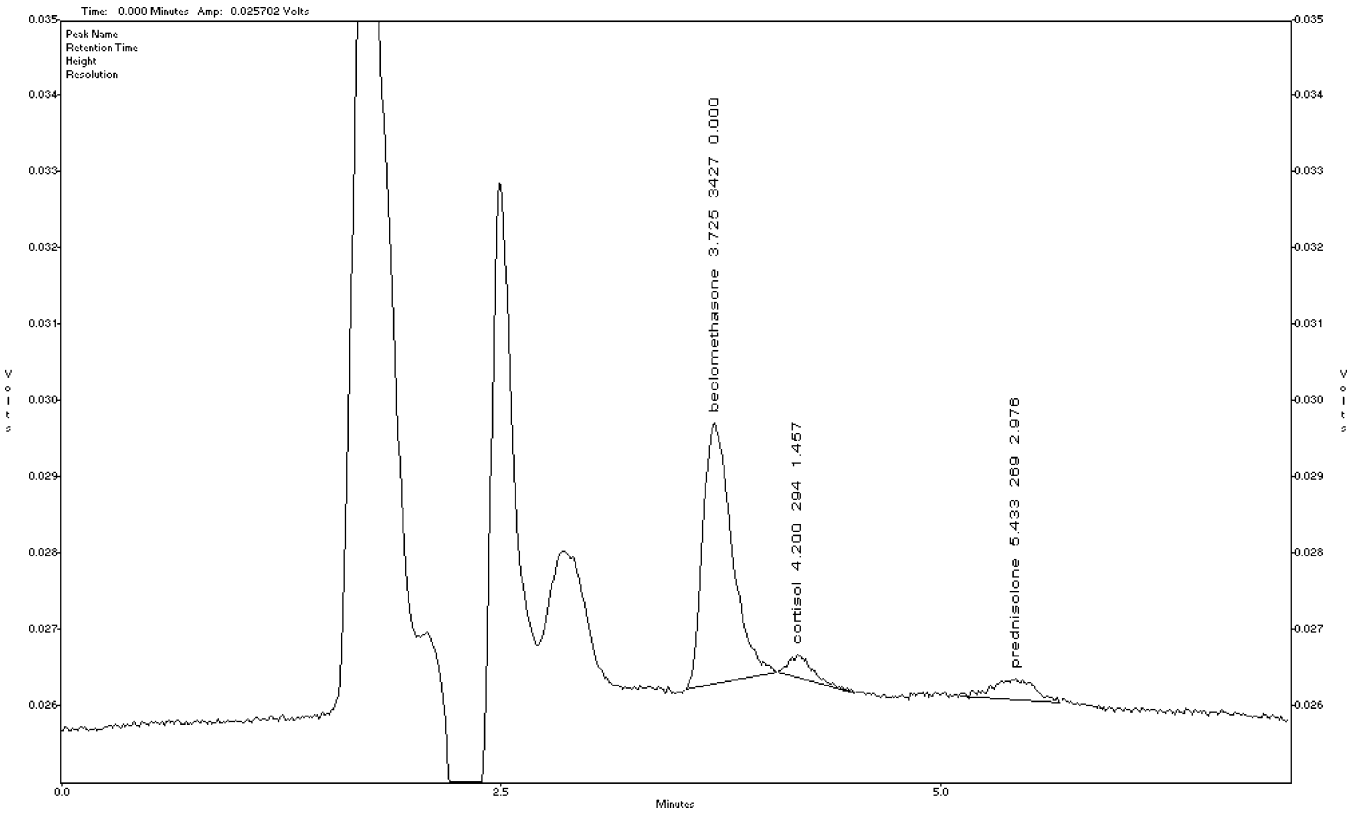

Fig. 5. A chromatogram of extracted urine spiked at 8 ng/ml prednisolone 7 ng/ml cortisol and 100 ng/ml beclomethasone.

3.2.6. Stability

The mean and standard deviation for the ratios between two measurements performed at a 3 month interval (storing at −70 °C) for plasma cortisol ($n = 5$), urinary cortisol ($n = 3$) and plasma prednisolone ($n = 3$) were: 0.98 ± 0.06, 1.1 ± 0.006 and 1.02 ± 0.05, respectively. This indicates that prednisolone and cortisol in plasma and urine samples are stable for at least 3 months when stored at −70 °C.

4. Summary and conclusion

In this article a valid and reliable method for routine analysis of prednisolone and cortisol in plasma and urine has been developed. The result was that the most effective washing (at step three of the SPE) for plasma samples is with 2% NH_4OH in 40% methanol and for urine samples 2% NH_4OH in 50% methanol.

The main advantage of this method over other published methods is the use of HLB copolymer SPE in the extraction process. Other advantages of this method over many other published methods is that washing was performed as a single step and that the method was specific for determination of prednisolone and cortisol in both plasma and urine with a total run time of 7.0–8.0 min.

By conducting an extensive literature search on the Medline (1966–2002) only three articles were cited in the literature for the determination of both prednisolone and cortisol in both plasma and urine [3–5]. However, these methods were limited by one or more of the following: the use of solvent extraction (SE), dependence on fluorimetric derivatisation or the use of three washing steps during the extraction process. Thus, although there were some aspects of these methods that were advantageous there were concomitant problems, e.g. the fluorometric assay had a lower limit of quantification [5] yet the retention time was up to 100 min.

Using the developed method it has been possible to analyse plasma and urine prednisolone and cortisol as a method for measuring adherence to prednisolone or high dose inhaled steroid therapy in 73 asthma patients [25].

Some patients' plasma samples blocked the cartridges; although, this did not happen frequently, it is advisable to centrifuge all the plasma samples before extraction to avoid this problem.

Baseline drift can happen frequently in normal phase chromatography; the best way to solve this problem is by running isopropanol for few min each time this happens [26].

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