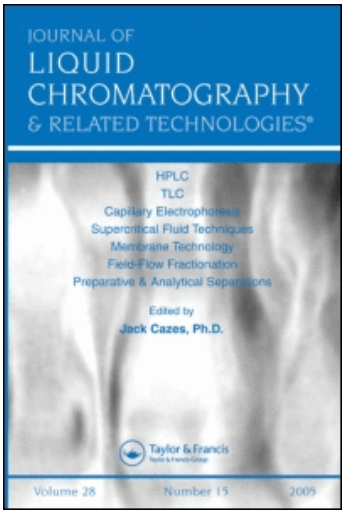


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# Use of Micellar Mobile Phases and an HPLC Column Switching System for Direct Injection Determination of Urinary Free Cortisol

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# USE OF MICELLAR MOBILE PHASES AND AN HPLC COLUMN SWITCHING SYSTEM FOR DIRECT INJECTION DETERMINATION OF URINARY FREE CORTISOL

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## ABSTRACT

A direct injection method for the determination of urinary free cortisol by an HPLC column switching system is presented. The first chromatographic column, which provides for sample preconcentration and clean–up, employs a micellar mobile phase. The second column, coupled on –line to the first, utilizes reversed–phase condition for analysis. UV detection is employed at 254 nm. Detection limit for urinary free cortisol is 2.4 ng when 2 ml urine sample is injected. Nine samples were analyzed. The results were in a reasonable range. This method can be used successfully in the diagnosis of patients with hypercortisolism and Cushing's syndrome.

## INTRODUCTION

High–performance liquid chromatographic (HPLC) methods applied in bioanalysis usually require elaborate sample pretreatment, including deproteinization, removal of interferences and sample enrichment. Traditionally, the methods of choice are liquid / liquid extraction or liquid / solid distribution, together with evaporation. But such procedures are labor–intensive and time consuming and the recovery is often low. A more practicable solution to this problem is the use of a simple direct injection technique.

The use of micellar chromatography has been reported to allow such direct injection of biological fluids (serum, plasma and urine) [1–4]. Micellar chromatography employs surfactants as mobile phase modifiers [5] to remove protein in untreated plasma / urine samples, but gives only moderate chromatographic efficiency due to poor mass transfer. Furthermore, the sensitivity of determination is inadequate for many applications.

In recent years column switching combined with the use of micellar clean–up prior to liquid chromatography has been introduced. Sodium dodecyl sulphate (SDS) is added to the mobile phase for loading the untreated plasma / urine samples onto a precolumn. The proteins are solubilized by the SDS and washed out, whereas the analyte is retained [6–8]. Next, the retained analyte is eluted with the analytical mobile phase and analysed using conventional RP–HPLC. This method has the advantages of both micellar chromatography(direct plasma / urine injection, extended column life and good recovery) and RP– HPLC ( high column efficiency).

Urinary free cortisol (UFC) is considered to be a specific and sensitive test for the diagnosis of Cushing's syndrome. Methods for determining UFC include competitive protein binding assays, radioimmunoassays (RIA) and high–performance liquid chromatography (HPLC). RIA procedures are designed primarily for the assay of serum cortisol, but urine contains many cross–reacting substances that make RIA of urine cortisol generally non–specific. Efforts to improve the specificity by extraction of cortisol with methylene chloride and washing the solvent extract with aqueous alkaline and acidic solutions were only partially success-

ful in removing the interferences [9]. Chromatographic separation of cortisol from interferring compounds is an efficient way of improving UFC assay specificity [10]. Schoneshofer and co–workers [11] quantified cortisol and other surinary steroids after collecting fractions separated by HPLC and assaying these fractions by RIA. AII these methods also involve the traditional tedious clean–up procedure before analysis and were unsuitable for routine clinical use.

In our laboratory, the column switching system was installed for the direct injection analysis of urinary free cortisol with a micellar mobile phase. The method is simple and rapid. The results are consistent with literature and reliable for clinical diagnosis.

## EXPERIMENTAL

### Apparatus

A diagram of the experimental set–up is shown in Figure 1. Solvent delivery was provided by two pumps. Pump 1(Model YSB–2, Shanghai Science Instrument Factory, China) was used for loading the sample onto the clean–up column(CC). Pump 2 (Model 6000A, Waters Assoc., Milford, MA, USA) was used to deliver the chromatographic mobile phase. The switching system consisted of a U6K injection valve ( Waters Assoc., Milford, MA, USA) with a 2ml sample loop and two K501 injection valves (Shanghai Science Instrument Factory, China). A Model 440 UV detector (Waters Assoc., Milford, MA, USA) was used for monitoring the effluent from the analytical column (AC). Data were acquired with a C–R2B chromatographic data system (Shimadzu, Kyoto, Japan).

### Column

The two column cartridges used in this study were manufactured by the Beijing Analytical Instrument Factory (Beijing, China) . Both the analytical column

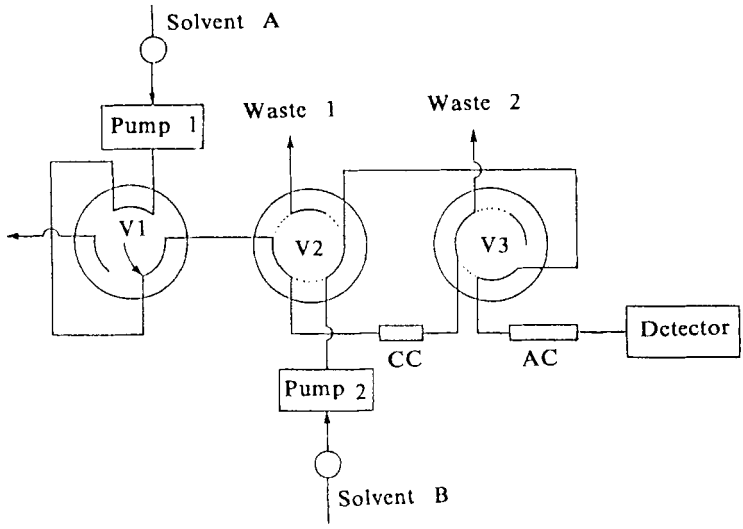


FIGURE 1. Schematic diagram of column switching system

(150 × 4.6 mm i.d., 5–μm spherical porous particles) and the clean–up column (50 × 4.6 mm i.d., 10–μm spherical porous particles) were packed with RP–18(Shanghai Chemicals Factory, China).

## Chromatographic Conditions

Solvent A: the micellar clean–up eluent; methanol / water / n–propanol (18 / 80 / 2 V / V / V) containing 20 mM SDS (pH = 6).

Solvent B: the analytical mobile phase; methanol / water / n–propanol(38 / 60 / 2 V / V / V) containing 20 mM SDS (pH = 6).

Flow rate: 1.0 ml / min.

Detection: UV absorption (254nm)

Injection volume: 2.0 ml.

### Chemicals

The chemicals used were obtained from a variety of suppliers. The standard sample of cortisol was obtained from The Second Hospital of Lanzhou Medical College.

## RESULTS AND DISCUSSION

### Assay Procedure

When mobile phase was delivered through the lines shown solid in Figure 1, solvent A (a weak eluent) was conveyed into CC by pump 1, the dissolved proteins and other weakly retained interferring urinary components were rinsed out from CC to waste W2, while the cortisol was still retained in CC. After eliminating protein, valves 2 and 3 were switched into the transfer position and mobile phase was delivered through the line shown dotted in Figure 1, cortisol was thus transferred from CC to AC by solvent B (a strong eluent). Then, valve 2 and 3 were rotated back, cortisol was eluted and separated by solvent B on AC. Finally, cortisol in urine were determined by UV detection and the next injection followed.

### Optimization of Chromatographic Condition and Switch Times

At first, an adequate analytical mobile phase ( solvent B) was chosen by orthogonal design tests. The concentration of surfactant, pH value and the concentration of organic solvent (methanol) are the three factors of orthogonal design and four levels of each factor were chosen. Tests for sixteen groups were conducted according to a $L_{16}(4^3)$ orthogonal table as shown in Table 1. The optimum result is 38% aqueous methanol containing 20 mM SDS with pH 6. To increase the column efficiency, 2% n–propanol were added.

The micellar clean–up eluent (solvent A) was tested as follows. As described above, it was necessary to rinse the clean–up column with a weak solvent to wash

**TABLE 1. The Factors and the Levels of the Orthogonal Design**

| Factor | Level | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| pH | 3 | 6 | 5 | 4 |
| $CH_3OH$(V / V) | 30% | 50% | 20% | 40% |
| [SDS](mM) | 10 | 20 | 30 | 40 |

out the proteins and other interferring urine components while the cortisol was retained in CC. We found that when 18% aqueous methanol containing 20 mM SDS was used as the weak solvent to flush CC at a flow rate of 1 ml / min, the protein and interferring components would be eleuted within 4 min, but cortisol was not washed out until 15 min. If 38% aqueous methanol was used, the cortisol was eluted at 2 min. Therefore, 18% aqueous methanol containing 20 mM SDS and 5 min were chosen as solvent A and switching time 1, respectively. Between 5 min and 10 min the whole of cortisol was delivered into AC. When after 10 min, valves 2 and 3 were rotated back, AC was eluted by solvent B while CC was again equilibrated by solvent A. This operating method not only minimizes the analytical time, but also avoids prolonged high back–pressure in chromatographic system.

Chromatograms of cortisol obtained in the above–mentioned chromatographic condition are shown in Figure 2. Figure 2a, 2b and 2c are chromatograms of cortisol standard solution, urine sample and urine sample + cortisol standard solution, respectively.

## Calibration Curve and Detection Limit

Using the chromatographic condition of Figure 2, the peak areas were plotted against the concentration of cortisol to give a calibration curve in which correla-

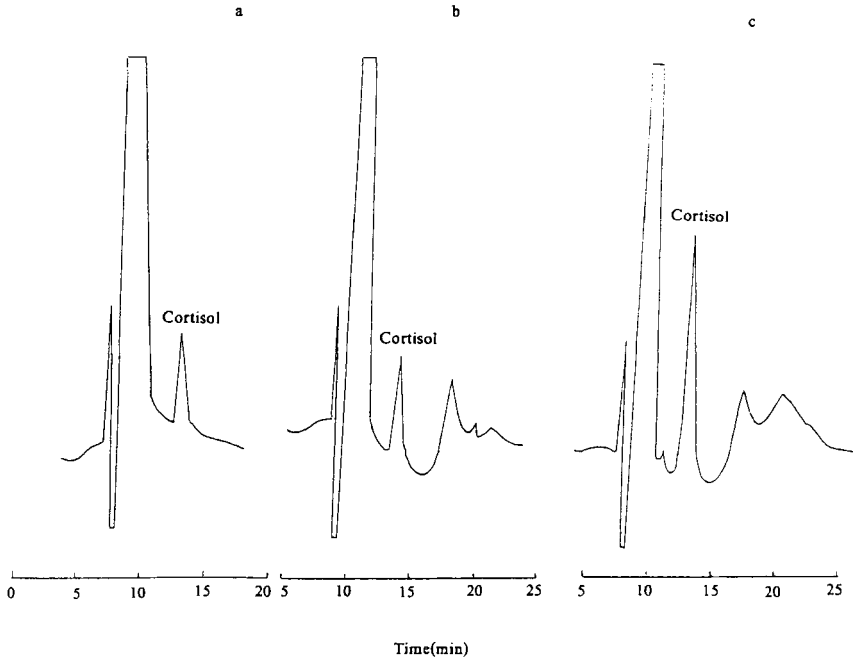


FIGURE 2. Chromatograms of UFC

a. Cortisol standard solution

b. Urine sample

c. Urine sample + Cortisol standard solution

tion coefficient is 0.997 and regression equation is:

$$Y = 3.57X + 0.714$$

The linear range of cortisol is 10–1000 $\mu g / L$, the detection limit is 2.4 ng. When the injection volume was 2 ml, the minimum detection concentration was 1.2 $\mu g / L$.

**TABLE 2. Recovery of UFC**

| Sample | B | S | Sm | Recovery(%) |
|---|---|---|---|---|
| 1 | 36.71 | 35.20 | 70.38 | 95.7 |
| 2 | 36.82 | 15.24 | 51.67 | 97.4 |
| 3 | 36.84 | 5.55 | 42.12 | 95.1 |

**TABLE 3. The Reproducibility of UFC**

| Peak Area | Relative Error (%) | R.S.D.(%) |
|---|---|---|
| 33.11 | −5.13 | |
| 36.82 | 5.50 | |
| 31.04 | −11.1 | 7.64 |
| 36.84 | 5.56 | |
| 36.71 | 5.19 | |

Recovery and Reproducibility of Cartisol

Different quantities of cortisol were added to urine samples and the recoveries calculated according to the following equation:

$$\text{Recovery} = [(S_m - B) / S] \times 100\%$$

where: S = the peak area given by a standard solution of cortisol;

B = the peak area of urinary free cortisol;

Sm = the total peak area after cortisol was added to the urine sample.

**TABLE 4. Analytical Results of UFC**

| No. of Sample | Sex * | Total Volume of 24–h urine (ml) | Amount of UFC ($\mu$g / 24–h) |
|---|---|---|---|
| 1 | f | 1450 | 10.40 |
| 2 | m | 1700 | 41.22 |
| 3 | f | 2300 | 56.11 |
| 4 | f | 800 | 4.123 |
| 5 | m | 2800 | 29.42 |
| 6 | m | 2450 | 14.49 |
| 7 | f | 3000 | 7.993 |
| 8 | f | 1200 | 11.43 |
| 9 | m | 2900 | 32.22 |

* m = Male, f = Female

The recoveries were 95. 1– 97. 4%, as shown in Table 2. The reproducibility was determined by an arbitrary sample. The Relative Standard Deviative (R.S.D) is 7.64% (n = 5) as shown in Table 3.

Determination of Urinary Free Cortisol

The concentration of urinary free cortisol is easily affected by drinking water and metabolite condition, it is changed a lot during one day. Therefore, a complete 24–hour urine sample was collected and a 25 ml of each urine sample was kept for use after the total volume was measured.

Using the regression equation of the calibration curve and correcting in terms of recovery, the amounts of free cortisol in 24 –hour urine samples were calculated

**TABLE 5. Result Ranges Compared with Literature**

| Method | Range of UFC | Literature |
|---|---|---|
| Present method | 4 – 56 μg / 24–h | |
| HPLC method | | |
| (Indirect Injection) | 0 – 37 μg / 24–h | [10] |
| RIA method | 47 – 110μg / 24–h | [13] |

according to the following equation:

$$UFC = (A - 0.714)\, V / 3.57 \times 96.1\%$$

where: UFC = the amount of the urinary free cortisol, μg / 24hour

A = the corresponding peak area

V = volume of 24–hour urine, liter

Numbers are the intercept and slope of the calibration curve and average recovery, respectively.

The results for the determinations of nine normal people's urine samples are listed in Table 4. Comparing the results that reported in literature as shown in Table 5, it is clear that the result range by this direct injection method is consistent with the ranges obtained by other HPLC methods. Generally ranges obtained by RIA are two to three times higher than those obtained using HPLC alone [12] or coupled HPLC– RIA method [11]. Above analysis conclusion is the same as that.

## CONCLUSION

We have developed a convenient direct injection HPLC method for the determination of urinary free cortisol utilizing a column switching technique. This

method is simple, rapid and accurate. It is suitable not only for the analysis of low level therapeutic drug in biological fluids [8], but also for the determination of endogenous substance.

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