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Original Article

HPLC determination and clinical significance of serum prednisone in patients with nephrotic syndrome

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Abstract: Aim: A rapid protocol is necessary to determine the serum concentrations of prednisone. Methods: The HP1100 high-performance liquid chromatographic (HPLC) system was employed. The HP Lichrosphere C8 column (250 mm × 4 mm, i.d., 5 μm particle size) was used. The mobile phase was methanol, tetrahydrofuran and water in the ratio 25:25:50. The flow rate was 1.0 ml/min. The sample was monitored by UV absorbance at 240 nm. Acetanilide was used as the internal standard, and methanol was added into the serum for depositing the protein. Results: The chromatography was effective and was not interfered with by the serum components. Good linearity was observed, within the range of 10-500 μg/L for prednisone, and the detection limit was 5 μg/L. The serum concentrations of prednisone between the nephrotic syndrome (NS) group and the control group were significantly different ($P < 0.05$), while there was no significant difference between the females and males of the NS group ($P > 0.05$). The serum ncentration of prednisone in the steroid-resistant group was lower than that in the steroid-sensitive group ($P < 0.05$). Conclusions: HPLC is a practical and reliable method to determine the serum concentration of prednisone with high accuracy, precision, linearity and repeatability.

Keywords: Drug monitoring, HPLC, prednisone

Introduction

Prednisone is widely used in the clinical treatment of nephrotic syndrome, hematopathy and dermatosis. Because the distribution, metabolic features and pharmacodynamics of prednisone in the human body have not been elucidated, the effective therapeutic concentration of prednisone is unknown. Thus, we cannot detect changes in the drug concentration or evaluate the curative effect. In addition, it is difficult to evaluate the differences in drug concentration among individuals. Furthermore, toxic side effects from megadoses or minimal efficacy from insufficient doses is inevitable. Accordingly, a rapid protocol is necessary to determine the serum concentration of prednisone. In previous studies, HPLC has shown great rapidity, exceptional accuracy and remarkable specificity in determining the serum concentration of prednisone. Moreover, HPLC is an effective method to monitor the curative effect on NS, which has important value for studying the curative effect on other diseases treated by the long-term administration of prednisone.

Materials and methods

Apparatus

The HPLC apparatus of the American Agilent HP1100 series included the following components: G1322 vacuum degassing system; G1311 Quatpump; G1316 constant temperature system; G1314 ultraviolet detector; American Agilent HP1100 chromatography workstation; domestic TGLL 18-table model, high-speed centrifuge; and XK-96A Whirlpool mixer.

Reagents

Methanol, tetrahydrofuran and ethyl acetate were chromatographically pure. A stock solu-

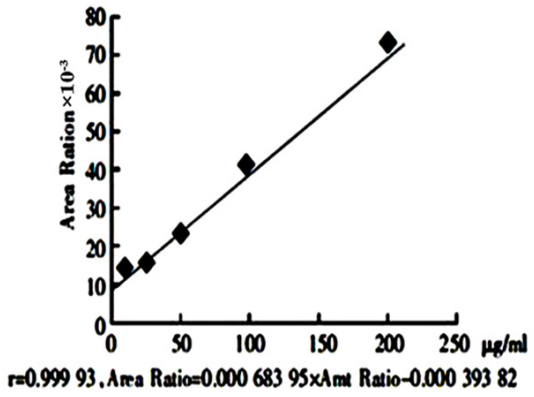

Figure 1. Standard curve. The regression equation is $Y = 6.84 \times 10^{-4}$-39.4×10^{-4}, and the correlation coefficient is 0.99993.

tion of the internal standard, namely, acetanilide (1 μg/ml) (Beijing Shuangxiangda Apparatus Business Department), was prepared in 95% methanol. The standard solution was prepared in 95% methanol with 8 μg/ml prednisone (Hunan Province Medicinal Materials Co., Ltd.).

Chromatography conditions

The mobile phase consisted of methanol, tetrahydrofuran, and water mixed in the ratio of 25:25:50 by volume. The column was thermostated at 25°C and operated at a flow rate of 1 ml/min. The detection wavelength was set at 240 nm, and the injection volume was 10 μl. The internal standard method was used to determine the concentration of prednisone. The total duration of the entire analysis was less than 10 min.

Sample sources

Ten NS patients diagnosed by renal biopsy were selected for the NS group from the Nephrology Department, Second Xiangya Hospital, Central South University. The NS group consisted of 7 minimal change disease (MCD) patients and 3 mesangial proliferative glomerulonephritis (MsPGN) patients as our observation subjects. There were 5 males and 5 females, aged from 18 to 62 years. In addition, 10 healthy volunteers were selected for the normal control group, which consisted of 5 males and 5 females aged from 25 to 30 years.

Sample collection

All the subjects were dosed with 50 mg prednisone orally (without taking any other drugs) at 8:30 a.m. Blood (3 ml) was collected from a venous indwelling needle (with heparin anticoagulation) into clean tubes at 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, and 10 h after prednisone administration. Next, the serum was separated by centrifugation. The serum was stored at -30°C if it was not immediately analyzed.

Sample processing

One milliliter of 5% perchloric acid was added to 1 ml of blood for complete precipitation of the protein, and the supernatant was separated by centrifugation. Fifty microliters of internal standard (1 μg/ml acetanilide methanol solution) was dispensed into the supernatant and mixed for 10 s. Next, 3 ml of ethyl acetate was added to the mixture and shaken for 10 min. The organic phase was transferred to another tube after centrifugation for 15 min at 4000 r/min. The organic phrase was evaporated to dryness under nitrogen stream at 45°C. The residue was reconstituted with 50 μl of methanol, and 10 μl of the solution was used for sample detection.

Statistical analysis

The paired t-test was employed to compare the averages of the normally distributed data; analysis of variance (ANOVA) was used to compare the interclass difference. All the analyses were performed using the SPSS 12.0 for Windows software.

Results

Standard curve and detection limit

Prednisone standard solutions at concentrations of 12.5, 25, 50, 100, and 200 μg/L were prepared with 5% perchloric acid, and 50 μl of the internal standard (1 μg/ml acetanilide methanol solution) was added. A 10 μl portion of the serum sample was analyzed in three replicates. Subsequently, the data were averaged to generate the standard curve. The least square method was used for the correlation regression analysis.

The standard curve for prednisone is shown in **Figure 1**. The concentration of prednisone was linearly related to the peak area ratio. The regression equation was $Y = 6.84 \times 10^{-4}$ X-39.4×10^{-4}, and the correlation coefficient was 0.99993 for prednisone. The lowest detectable

Table 1. The concentrations of Prednisone standard solutions times (n=3, x±s)

Times	Concentration Gradient of Prednisone standard solutions (μg/mL)				
	12.5	25	50	100	200
1	12.36	24.65	49.42	98.68	197.36
2	12.31	24.69	49.32	98.72	197.45
3	12.32	24.7	49.31	98.76	197.39
Mean	12.33	24.68	49.35	98.72	197.4
SD	0.026	0.026	0.061	0.040	0.046

concentration of prednisone was 5 μg/L (signal/noise ratio = 3) (**Table 1**).

Chromatograms of the standard substance

Figure 2 displays the chromatograms of the standard substance (prednisone, 80 μg/L; acetanilide, 10 μg/L). The retention times for prednisone and acetanilide were 6.230 min and 2.746 min, respectively. Prednisone and acetanilide reached the baseline separation and were totally separated.

Precision

For the intra- and interassay precision studies, we prepared drug-free serum with concentrations of 8 μg/L prednisone and 10 μg/L acetanilide. Next, the serum was divided into 20 parts for detection; the calculation yielded a result of 8.03±0.18 (mean ± SD). The intraassay coefficient of variation (CV) was 2.25%. Subsequently, an additional 20 samples, which had been frozen-preserved, were detected every day; the mean ± SD was 7.96±0.03, and the interassay coefficient of variation (CV) was 3.76%.

Recycling test

Fifty-microliter aliquots of prednisone standard solutions with concentrations of 200 μg/L, 150 μg/L, and 50 μg/L, respectively, were each added to 450 μl of normal serum (the protein was precipitated by 5% perchloric acid) as the analytical samples. Fifty microliters of 5% perchloric acid was added into the serum as the basic sample. Next, 10 μl of the processed sample was injected for analysis. Each sample was determined in three replicates, the average was calculated, and the recovery was 98.7% (**Table 2**).

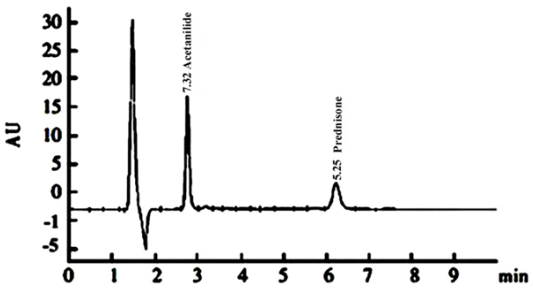

Figure 2. Chromatograms of the standard substance (prednisone, 80 μg/L; acetanilide, 10 μg/L).

Interference test

With the same conditions of the mobile phrase, chromatographic column and flow rate, several commonly used drugs (such as vitamin C, a tranquilizer, paracetamol, and chlorpromazine) were added to the serum samples that were formerly prepared to evaluate the precision. The retention times for prednisone, acetanilide, vitamin C, the tranquilizer, paracetamol, and chlorpromazine were 6.245 min, 2.746 min, 1.887 min, 5.195 min, 7.298 min and 1.856 min, respectively. Each interferent was well extracted with prednisone and acetanilide, which indicated that no interference from these drugs affected the determination results under the test conditions.

Results of the serum prednisone concentrations

Table 3 demonstrates that the serum concentrations of the NS group and the control group were significantly different ($P < 0.05$), whereas the serum concentrations in males and females of the NS group exhibited no significant differences ($P > 0.05$).

Analysis of the serum drug concentrations and curative effects in NS patients with different pathological types

Ten primary nephrotic syndrome patients were selected, including 7 MCD patients and 3 MsPGN patients; these subjects were administered prednisone separately for pulse therapy (at a dose of 55-60 mg). The 2 h serum concentrations and urinary protein were investigated separately before and after the prednisone administration; in addition, the serum concentrations, changes in urinary protein and curative effect in the steroid-resistant and steroid-

Table 2. Recovery rate of prednisone with different concentrations (n=3)

Basic concentration (μg/L)	Intrant	Detecting	Recovery (%)
	Concentration (μg /L)	Concentration (μg /L)	
50	50	49.35	98.7
50	150	147.90	98.6
50	200	197.60	98.8

sensitive patients were determined (**Tables 4** and **5**).

Discussion

In this research, we employed acetanilide with hypotoxicity and higher extraction efficiency; the mobile phrase was filtered and air-exhausted on a Lichrosphere C8 column (250 mm × 4 mm i.d., 5 μm particle size). Meanwhile, the sample had been completely deproteinized with 5% perchloric acid before being processed. Baseline separation was achieved between prednisone and acetanilide, and these substances were completely separated. The mean recovery and inter-day CV were 98.7% and 3.76%, respectively. We have used the ultraviolet spectrum to detect prednisone from 90 nm to 400 nm wavelength. Our study revealed that the samples' maximum absorption peaks appeared at a wavelength of 240 nm, under which the best separation effect was observed; therefore, 240 nm was selected as the determination wavelength. The lowest detectable concentration of prednisone was 5 μg/L, which improved the linear range for detection with good repeatability.

Through the interference test, we found that (under the same conditions of the mobile phrase, chromatographic column and flow rate), compared with prednisone and acetanilide, the retention times of vitamin C and chlorpromazine were similar to acetanilide, and the separation effect was superior. The test revealed that the tranquilizer and paracetamol did not affect the determination of prednisone.

Prednisone is a major hormonal drug used for treating NS, and this medicament functions only when converted into prednisolone in the liver. The pharmacokinetics of prednisone and prednisolone in the body is a complex pathway with a reversible metabolism and a nonlinear elimination. Currently, there is no pharmacokinetics model that entirely reflects the complex mechanism of prednisone and prednisolone in vivo. Animal experiments have confirmed that there are two fundamental characteristics of the pharmacokinetics of prednisone and prednisolone, namely, dose-dependence and reversible metabolism [1]. These complex mechanisms of prednisone and prednisolone in vivo render pharmacokinetic research rather difficult; thus, research into these compounds is mainly focused on animal experiments, and human studies are rare. Hydrocortisone mainly combines with glucocorticoid receptors in the cytoplasm and then functions after being transferred into the nucleus. The functional potency is determined by receptor affinity and free hormone at receptor sites, which are influenced by many pharmacokinetic factors [2]. However, prednisone is well absorbed orally. By detecting the serum concentrations in the NS patient group, we found that the concentration of prednisone was detected earliest at 0.5 h after the prednisone administration, reached its peak after approximately 2 h and then declined. The results show that the average concentration of prednisone does not significantly differ between males and females ($P > 0.05$). We used the assay described herein to detect the prednisone concentrations in 10 normal individuals; the peak concentration was close to that in the published report, namely, 43.0±11.8 μg /L [3]. However, the average, peak prednisone concentration in the NS group was significantly higher than in the normal group, which indicates that the metabolic rate of the NS group was lower than that of the normal control group. The reason might be the difference in pharmacokinetics between NS patients and normal individuals; whether or not this phenomenon is related to taking prednisone combined with other drugs must be further studied, using a larger sample size.

The sensitivity of hormonal therapy for NS patients is nearly 60%, but the recurrence rate is higher than 60%. Studies have revealed that these factors are mainly related to different pathological types, age, gender and other characteristics. By analyzing 7 MCD patients and 3 MsPGN patients, we found that of the 7 MCD patients tested, five were sensitive to corticosteroids, two were insensitive, and the sensitiv-

Table 3. The serum prednisone concentrations of two groups at different times (x±s)

Groups		Time (h)							
		0.5	1	2	3	4	5	7	10
NS	male	18.28±15.36[a]	36.32±17.46[a]	58.95±19.36[a]	46.05±18.27[a]	30.68±19.55[a]	26.74±16.55[a]	19.87±10.29[a]	15.32±11.27[a]
	female	19.22±17.51[a]	34.58±22.35[a]	62.31±14.55[a]	44.11±19.37[a]	28.33±22.54[a]	24.45±14.23[a]	21.54±10.30[a]	17.55±10.28[a]
Control	male	12.36±9.67	25.48±12.15	38.45± 13.47	32.24±12.57	24.17±13.36	19.11± 10.26	15.85±11.45	7.69±12.43
	female	15.21±11.25	24.37±13.54	41.20±14.55	29.58±16.66	25.8 9±12.22	22.30±11.45	17.54±10.12	8.58±11.67

[a]Compared with control group: $P < 0.05$.

Table 4. Serum concentrations and curative effects for NS patients with different pathological types

Pathological types	serum concentration (μg/L)	Changes of urine protein		Curative effect
		Prior to treatment	After treatment	
MCD	52.31	6.88	1.65	sensitive
MCD	67.52	7.44	2.15	sensitive
MCD	72.35	5.40	1.05	sensitive
MCD	53.21	3.60	1.70	sensitive
MCD	67.81	4.02	0.35	sensitive
MCD	53.24	7.60	6.90	resistant
MCD	48.39	4.10	4.35	resistant
MsPGN	50.45	13.57	14.32	resistant
MsPGN	68.56	4.86	1.03	sensitive
MsPGN	72.51	3.34	1.35	sensitive

ity was 71.4%. Of the 3 MsPGN patients tested, only one was sensitive to corticosteroids, two were insensitive, and the sensitivity was 33%. In addition, based on **Table 5**, the 2 h peak serum concentrations indicated that the PS serum concentrations in the steroid-sensitive patients were significantly higher than in the steroid-resistance patients, and this difference was statistically significant. There was an extremely significant difference in the amount of urinary protein before and after taking prednisone in the steroid-sensitive group, but there was no statistical significance in the steroid-resistant group. Because we did not determine the base-level serum concentrations before the prednisone administration, whether or not the different results are related to the low metabolic rate of the serum PS concentration in the steroid-resistant subjects must be affirmed by further study.

Furthermore, the administration of prednisone usually incurs complications. For instance, long-term usage can exacerbate infectious diseases of the kidney. Because of the decreased metabolic clearance of insulin and C-peptide in the kidney, after taking prednisone, the abnormal glucose tolerance is likely to be superimposed by steroid diabetes, a delayed C-peptide peak, a decreased peak value and increased urinary glucose. In addition, hyperlipidemia is the main clinical indication of NS and is the critical cause of the high morbidity of atherosclerosis and myocardial infarction [4, 5]. Related studies have demonstrated that hyperlipidemia may directly lead to glomerular sclerosis and may accelerate renal failure [6]; thus, disorders of lipid metabolism should be avoided and rectified along with the NS therapy. Thus, detecting the serum prednisone concentration during prednisone administration facilitates assessment of the effects on multiple system functions. If the concentration of endogenous GCs (such as hydrocortisone) in the blood could be monitored simultaneously, there would be important clinical significance for observing the curative effect of GCs, studying the influence and interaction of exogenous GCs in the metabolism of endogenous GCs and the relationship to the curative effect of GCs. With continuous improvement of the experimental conditions, we can combine the detection of the peak concentration and trough concentration of prednisone, and we can continuously and dynamically monitor the treatment, which may more objectively reflect the pharmacokinetic and pharmacodynamic characteristics of prednisone and preferably direct the clinical medication and avoid the onset of complications.

In this study, we just analysis 10 patients. The sample size is indeed less, but the data statistically significant. However, the sample size should be expanded.

Table 5. Comparison of serum concentrations and curative effect for steriod-resistance and steriod-sensitive

Sensibility of steriod	N	plasma Concentration (μg/L)	Urine protein content		Curtive effect
			Prior to treatment	After treatment	
Sensitive NS	7	64.89±16.74[a]	5.07±3.13	1.33±1.13[b]	sensitive
Resistant NS	3	50.69±4.78	8.42±0.54	8.52±10.15	resistant

[a]compared with sensitive NS group, $P < 0.05$. [b]compared with premedication, $P < 0.05$.

With the continuous increase of drug-type monitoring and the increasing popularization of chromatographic techniques, clinical pharmacokinetics monitoring (CPM) has made a positive contribution to clinical therapy and scientific research and has been widely valued in clinical practice. Through more than twenty years of development, domestic TDM has begun to play an irreplaceable role in improving the level of rational clinical management. Moreover, with the research progress in the relationship between serum concentrations and curative effects, pharmacokinetic research (which uses pharmacodynamics as an index) will emerge as a new, practical field [7-11] and will play an important role.

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Disclosure of conflict of interest

None.

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