Journal of Chromatography, 305 (1984) 194—198
Biomedical Applications
Elsevier Science Publishers B V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1905

Note

Application of a high-performance gel permeation liquid chromatographic procedure to the determination of binding of prednisolone to high-affinity binding sites in human serum

J.C.K. LOO*, N. JORDAN and A. HO NGOC

Bureau of Drug Research, Health Protection Branch, Health and Welfare Canada, Ottawa, Ontario K1A 0L2 (Canada)

(Received June 21st, 1983)

It has been established that prednisolone, a synthetic corticoid, binds to corticosteroid-binding globulin (CBG) and albumin (AB) in human serum [1—4]. The binding affinity of the steroid for CBG is high ($K = 3 \cdot 10^7$ ml^{-1}) whereas the capacity is low. In contrast, albumin has a low affinity ($K = 2 \cdot 10^3$ ml^{-1}) for the drug but the binding capacity is high. Various authors [1—3] have attempted to evaluate the binding characteristics of the drug to albumin and CBG in serum by equilibrium dialysis and by employing non-linear regression analysis which is based on an appropriate mathematical model. Nevertheless, there is no report of a direct measurement of the binding of the drug to high-affinity binding sites in serum. Hoffman and Westphal [5] have employed gel permeation chromatography (Sephadex) for the evaluation of binding of hydrocortisone (HC) to CBG in plasma. This is based on the fact that HC bound to AB completely dissociates during the chromatography described. In contrast, HC bound to CBG dissociates slowly on the column. The method, however, is tedious.

This report describes a high-performance liquid chromatographic (HPLC) gel permeation procedure which allows prednisolone bound to AB to completely dissociate during chromatography while the binding of the drug to high-affinity proteins is unaffected.

EXPERIMENTAL

Materials

Prednisolone (U.S.P. reference) and fatty acid free human serum albumin

0378-4347/84/$03.00

(Sigma, St. Louis, MO, U.S.A.) were used for the preparation of solutions. Prednisolone [6,7-^{3}H (nominal)] with a specific acitivity of 1.96 terabecquerels/mmol (New England Nuclear, Montreal, Canada) was used. HPLC solutions were prepared from double-distilled water. Spectrapor 2 membrane tubing (Spectrum Medical Industries, Terminal Annex, Los Angeles, CA, U.S.A.) and 1-ml dialysis chambers (Technilab Instruments, Pequamok, NI, U.S.A.) were used in the equilibrium dialysis studies.

Chromatographic procedure

The constant-volume HPLC system consisted of a pump (Altex Model 110A, Berkeley, CA, U.S.A.), an injector (Rheodyne, Berkeley, CA, U.S.A.) fitted with a 20-μl loop and a UV detector (Waters 440, Milford MA, U.S.A.) set at 280 nm. The column (Bio-Sil TSK-250, 300 × 7.5 mm, particle size 10 ± 2 μm, Bio-Rad Labs., Richmond, CA, U.S.A.) with a molecular mass range of 1000—300,000 was preceded by a guard column (O-SIL, TSK ARD, Bio-Rad Labs.). The chromatographic system was operated at 4°C by placing the mobile phase and column in an ice bath. After use, the column was washed with 0.05% sodium azide and stored at 4°C. The mobile phase consisted of 0.1 *M* sodium sulfate and 0.02 *M* sodium phosphate monobasic adjusted to pH 6.8 with 0.1 *M* sodium hydroxide. Degassing was accomplished by means of a stream of helium bubbled through the mobile phase. A flow-rate of 54 ml/h was used.

General procedure

Aliquots (25 μl) of serum spiked with [^{3}H] prednisolone and cold carrier were placed in glass culture tubes, covered and incubated in a water bath at 37°C for 3 h. Following incubation, the sample was diluted with 1.0 ml mobile phase (at 4°C); 20 μl of the sample were immediately chromatographed. The column eluent was collected stepwise at 1-min intervals and the radioactivity was determined in a beta-counter. Periodic injections of a standard protein mix containing thyroglobulin, chicken ovalbumin, bovine myoglobin, phenylalanine and cyanocobalamin which was supplied by the column manufacturer, were used to monitor column performance.

Dialysis procedure

Incubates of serum containing [^{3}H] prednisolone with carrier prednisolone at a concentration range of 50—800 ng/ml were placed in dialysis chambers separated by dialysis membranes and dialysed against an equal volume of 0.054 *M* phosphate isotonic buffer, pH 7.4. The cells were shaken in a water bath at 37°C for 3 h. Preliminary studies had shown that equilibrium was achieved in this time.

The study of the binding of prednisolone to albumin was carried out under similar conditions. The albumin concentration in buffer was 4% which is the concentration in normal human serum.

RESULTS AND DISCUSSION

Fig. 1a depicts a chromatogram following the injection of an aliquot of prednisolone (200 ng/ml) incubated in 4% albumin. It is evident from the

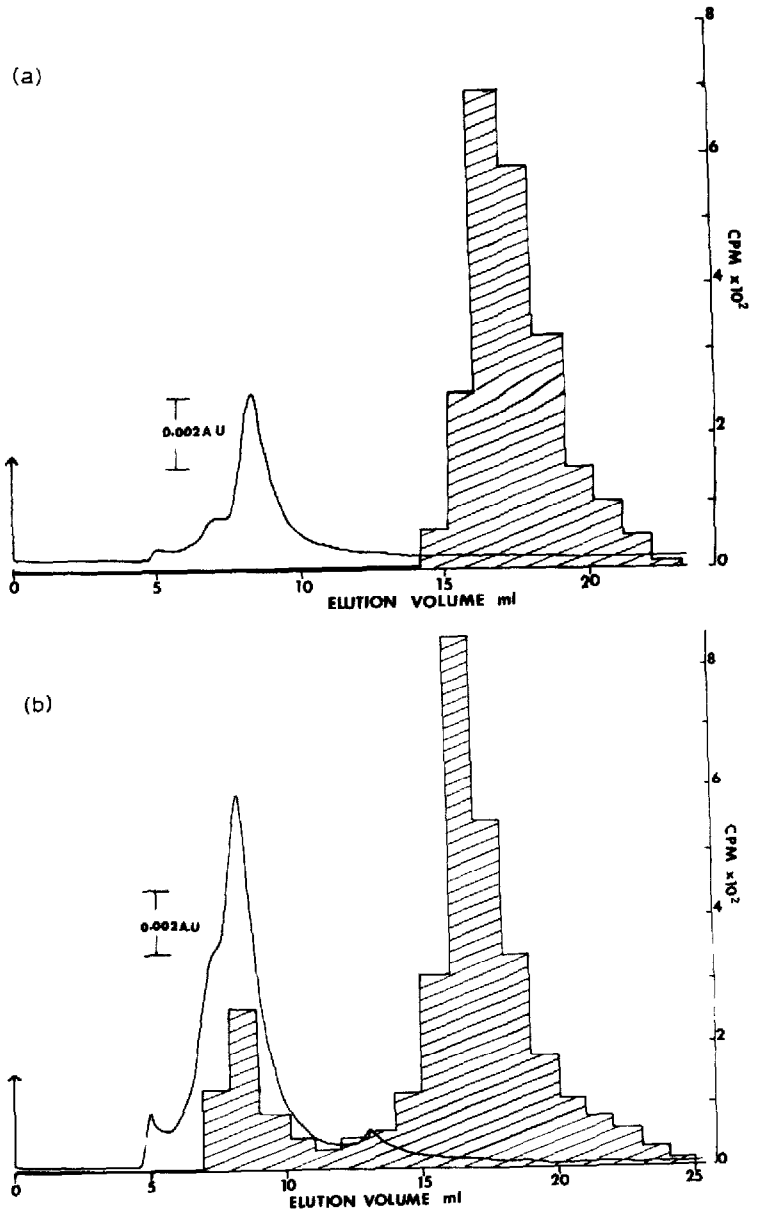

Fig. 1. Chromatograms following injection of an aliquot of prednisolone (200 ng/ml), (a) incubated with albumin (4%) and radioactive tracer, and (b) incubated with serum and radioactive tracer The histogram represents the radioactivity.

histogram that the drug bound to albumin is completely dissociated during HPLC. Similar results were obtained at lower prednisolone concentrations (25 ng/ml).

Fig. 1b illustrates a chromatogram of prednisolone (200 ng/ml) incubated in human serum. Some of the radioactivity was found in the protein fraction as well as in the prednisolone region. In the former region, the drug is probably

TABLE I

EFFECT OF PREDNISOLONE CONCENTRATION ON THE BINDING IN SERUM (HPLC)

Concentration (ng/ml)	Percent bound	
	Subject 1	Subject 2
25	14.5	26.9
50	13.3	24.9
100	12.5	24.2
200	9.69	20.2
400	8.25	15.1
800	7.5	12.2

TABLE II

COMPARISON OF BINDING OF PREDNISOLONE BY HPLC AND EQUILIBRIUM DIALYSIS

Prednisolone (ng/ml)	HPLC	Equilibrium dialysis	
	Percent bound to high-affinity protein	Percent bound to serum	HPLC value + percent bound to albumin*
50	29.1	87.6	80.4
100	28.1	86 4	79.4
400	19.7	73.3	71.0
800	13.9	65.7	65.2

*The percent bound to albumin as determined by equilibrium dialysis is 51.3% and is independent of concentration This value is added to the HPLC value.

bound to a high-affinity and low-capacity binding protein such as CBG. The effect of flow-rate on the binding of prednisolone in serum was studied. The amounts bound were found to be constant in the 0.8—1.5 ml/min range, indicating that the drug is not dissociated from this high-affinity binding protein during HPLC.

The relationship between the degree of binding in serum and drug concentration as determined by HPLC, is described in Table I. The concentration-dependent binding noted has also been reported by other authors [1—4].

Replicate analyses of serum containing 25 ng/ml prednisolone demonstrated the reproducibility of the method (C.V. = 4.6%, n = 6). With a concentration of 100 ng/ml, prednisolone binding to serum from six volunteers (HPLC) was found to be 16.97%, C.V. = 32.8. The range was between 8.1 and 23.1%.

Binding values determined by HPLC were compared to results obtained by equilibrium dialysis and are tabulated in Table II. Although it is recognized that amounts bound are not additive, the HPLC values plus albumin are in reasonably good agreement with the total serum binding.

CONCLUSION

In conclusion, by using the conditions described, the drug bound to albumin is completely dissociated during the chromatographic procedure. In contrast, when serum is chromatographed, the percentage bound drug is independent of flow-rate, which indicates that the drug is bound to a high-affinity binding protein such as CBG and does not dissociate appreciably in the experiment. This HPLC gel permeation provides a simple method for the direct measurement of specifically bound drugs. This method should be applicable to the study of binding of other steroids to high-affinity proteins in serum.

REFERENCES

1 J.G Wagner, D. Wexler, I.T. Agabeyoglu, R.F. Bergstrom, E Sakmar and D.R. Kay, J. Lab. Clin. Med., 97 (1981) 487.
2 M L Rocci, Jr., R. D'Ambrosio, N.F. Johnson and W.J. Jusko, Biochem. Pharmacol., 31 (1982) 289.
3 F.J. Frey, J.G. Gambertoglio, B M. Frey, L Z Benet and W.J C. Amend, Eur. J Clin. Pharmacol , 23 (1982) 65
4 G.P Lewis, W.J. Jusko, C.W. Burke and L. Graves, Lancet, ii (1971) 778.
5 W Hoffmann and U Westphal, Anal. Biochem., 32 (1969) 48.