Determination of Cortisol in Human Plasma by Thin-Layer Chromatography and Fluorescence Derivatization with Isonicotinic Acid Hydrazide

Martin Fenske*
Department of Animal Physiology, University of Bayreuth, 95440 Bayreuth, Germany

Abstract

The present work describes a specific and rapid determination of cortisol in human plasma. The method includes liquid–liquid extraction of plasma samples, thin-layer chromatography (TLC) of ethanolic extracts on aluminium foil-backed silica gel 60 TLC plates, derivatization of cortisol with isonicotinic acid hydrazide, and densitometric measurement of the fluorescence intensity of cortisol hydrazone. The fluorescence was linearly related to cortisol amounts; the correlation coefficients of standard curve plots were $r > 0.99$. The coefficient of variation ranged between 2.8–7.9% (20 ng, within-assay/between assay variation) and 1.6–6.8% (80 ng, within-assay/between assay variation). The recovery of cortisol from plasma spiked with 21-deoxycortisol was 85% ± 4%. Cortisol concentration in the plasma was 66 ± 32 ng/mL (mean ± standard deviation, $n = 24$). The advantage of this method is its simplicity to separate cortisol from other steroids by TLC, its specificity (formation of cortisol hydrazone), and the rapid quantitation of cortisol by densitometry.

Introduction

Isonicotinyl hydrazones of numerous ketosteroids were prepared in 1952 by Ercoli et al. (1). On the basis of this and similar investigations, several methods have been developed for estimation of Δ-4-ketosteroids in biological (2–4) or pharmaceutical material (5–8). However, they were not validated or used as routine methods for cortisol (F) measurement in human plasma because they might have been considered to lack sensitivity, specificity, and/or convenience of analysis. Thus, the aim of this work was to describe a rapid and specific routine method for the determination of F in human plasma, which is attractive because of its simplicity to separate F from other substances (thin-layer chromatography) (TLC), its specificity (fluorescence of isonicotinyl hydrazone), and its rapid quantitation (densitometry).

* Author to whom correspondence should be addressed: e-mail martin.fenske@uni-bayreuth.de.

Experimental

Materials

Aluminium foil-backed silica gel 60 TLC plates (20 × 20 cm with concentrating zone, F_{254}), technical grade dichloromethane, and other solvents and chemicals of analytical grade were supplied by VWR International (Darmstadt, Germany). Steroids were supplied by Sigma (Taufkirchen, Germany), and chicken serum was supplied by Serva (Heidelberg, Germany). Human plasma samples were donated by the central laboratory of a local hospital in Bayreuth; they were taken in the early morning (08.00–10.00 h, $n = 34$).

Extraction of samples, chromatography, and F measurement

Extraction of F from plasma

One-tenth of a milliliter of internal standard (21-deoxycortisol, 100 ng) was added to 0.5-mL portions of each plasma (duplicate). Samples were mixed and transferred to kieselguhr-filled minicolumns (9). Steroids were extracted with 3 mL dichloromethane; after evaporation of the solvent, the dry residue was reconstituted in 150 µL of ethanol, with careful rinsing of the glass walls.

TLC, F measurement

Samples (100 µL) and F standards (20, 40, and 80 ng) were applied to a series of points on TLC plates using an autospotter device (Linomat IV, CAMAG, Berlin, Germany). Samples were batch-analyzed to avoid variation between plates (8 lanes: sample extracts, 4 lanes: F standards); they were spotted in 8 mm bands 15 mm from the lower edge of the concentrating zone. With the exception of the preliminary tests (Table I), plates were developed by ascending chromatography in the solvent acetone–toluene (1:1, v/v). In all cases, plates were developed for a distance of 150 mm. They were first scanned at 254 nm (measurement of fluorescence quenching) to detect steroids and caffeine. The plates were then dipped into the isonicotinic acid hydrazide (INH) reagent (4). To further increase and stabilize the fluorescence of the F hydrazone, 60 min after being dipped into INH, plates were

dipped for 2 s into liquid paraffin oil–dichloromethane (1:5, v/v). The fluorescence intensity of the F isonicotinyl hydrazone was measured by a CAMAG TLC Scanner II (CAMAG). Peaks were integrated by means of Cats 3 computer software (CAMAG). The settings of the scanner were: excitation wavelength, 370 nm; emission wavelength, > 460 nm; monochromator bandwidth, 30 nm; slide length, 12 mm; and slide width, 1.2 mm.

Statistics

Data are expressed as mean ± standard deviation (SD). Correlations between parameters were assessed by linear regression analysis.

Results

Successful separation of F from cortisone or caffeine was achieved on aluminum foil-backed silica gel 60 TLC plates using solvent systems that consisted of two or more of the following solvents: toluene, chloroform, dichloromethane, acetone, methanol, and water (Table I). Acetone–toluene (1:1, v/v) was found most reliable, both with respect to the reproducibility of chromatograms and the separation of F from other substances. It may be worth mentioning that synthetic glucocorticosteroids like fludrocortisone or dexamethasone could not be separated from F in these solvents, supporting a finding reported in the literature (10,11). Table II shows that the fluorescence of F hydrazone was linearly related to F amounts; the correlation coefficients of standard curve plots (peak area against amount of F applied) were $r >$ 0.99. The coefficient of variation (CV) ranged between 2.8%–7.9% (20 ng, within-assay/between-assay variation) and 1.6%–6.8% (80 ng, within-assay/between-assay variation). The limit of detection was determined by viewing, under 366 nm, the INH-treated plates containing 10, 20, 40, or 80 ng of F. The band of 10 ng was not visible, but the zone from the 20 ng was barely visible. Thus, the limit of F detection was taken as 20 ng. 21-Deoxycortisol (21-DF) was used as internal standard because 21-DF and F were effectively removed from the plasma by a simple extraction step; 21-DF was well separated from F by a single development in acetone–toluene (1:1, v/v), and 21-DF and F peaks could be scanned and quantitated simultaneously. The recovery of 21-DF and F from a steroid-stripped, 21-DF- and F-spiked human plasma (200 ng/mL) was 85% ± 4 (21-DF, n = 10) and 83% ± 5 (F), respectively. When F (20, 40, or 60 ng, triplicates) was added to charcoal-treated human plasma (endogenous F content: 6 ng/mL), the F concentration was 22 ng/mL, 38 ng/mL, and 55 ng/mL, and the recovery was 85%, 83%, and 83%, respectively. A pool of human plasma spiked with 100 ng/mL F was used for the linearity test. The F content of the steroid-spiked, undiluted plasma was 164 ng/mL. Serial dilution of the plasma (1:1, 1:3, 1:7, v/v) gave a linear correlation between expected and measured F amounts (r = 0.95, n = 4). Another pool of plasma (charcoal-stripped plasma, spiked with 50 or 100 ng/mL) was used to assess precision, and the within-day variation of estimates (n = 8, one plate) was 61 ± 3 ng/mL [coefficient of variation (CV) = 4.9%] and 108 ± 4 ng/mL (CV = 3.7%). The between-day variation of estimates when the samples were measured as duplicates on 10 plates at 2–4 day intervals was 64 ± 5 ng/mL (CV = 7.0%) and 113 ± 8 ng/mL (CV = 7.1%). The F concentration in plasma samples donated by the central laboratory of a local hospital in Bayreuth was 66 ± 32 ng/mL (n = 20). The very same results were obtained after the plates were scanned 2 h (69 ± 29 ng/mL) or 24 h (65 ± 33 ng/mL) after dipping into INH, indicating the stability of the fluorophore (F hydrazone, Table II B).

Table I. Thin-Layer Chromatography and R_f Values of Steroids and Caffeine Using Different Solvent Systems*

	Solvent system			
Steroid	I	II	III	IV
cortisol	0.17	0.36	0.13	0.17
caffeine	0.19	0.24	0.37	0.42
dexamethasone	0.19	0.37	0.13	0.21
cortisone	0.26	0.42	0.23	0.31
corticosterone	0.26	0.43	0.28	0.36
11-deoxycorticortisol	0.32	0.52	0.31	0.41
21-deoxycortisol	0.40	0.55	0.27	0.39

* Note that a difference of 0.03 in R_f values well-resolves two bands from each other. Solvent system I: acetone–toluene–chloroform (1:1:1, v/v/v 4); solvent system II: acetone, toluene (1/1/1, v/v/v 4); solvent system III: dichloromethane, methanol, water (150/9/0.2, v/v/v16); solvent system IV: chloroform, ethanol, water (90/10/2, v/v/v17).

Discussion

The TLC–INH method of F analysis in human plasma is similar to that used for guinea pig plasma (3). With respect to possible interfering substances in human plasma (e.g., caffeine),

Table II. Standard Curve for F Measurement by TLC–INH Method: Within- and Between-Assay Precision (a), and Stability of the F Hydrazone Fluorescence (b)

	Cortisol (ng)		
	20	40	80
a. Precision			
within-assay			
mean	424*	899	1854
SD (n = 3)	± 12	± 26	± 30
between-assay			
mean	454*	921	1899
SD (n = 3)	± 36	± 61	± 129
b. Stability of F hydrazone			
fluorescence (n = 2)			
reading after 1 h	434	911	1847
readings after 2 h	431*	904	1844
readings after 24	429*	911	1821

* Integrated peak values (arbitrary units). F standards (20–80 ng) were applied to TLC plates. Plates were developed in acetone–toluene (1:1, v/v), dried in air, and then dipped into INH and one hour later into liquid paraffin oil–dichloromethane (1/5, v/v). Plates were scanned 1 h, 2 h, and 24 h after dipping into INH. Fluorescence intensity was measured densitometrically (excitation wavelength: 370 nm, emisson wavelength: 460 nm).

it was necessary to use other solvent systems than acetone–toluene–chloroform (see retention factor values in Table I), for example, acetone–toluene. The specificity of the method presented here is also given by the specific reaction of INH only with ketosteroids having a double bond conjugated with a carbonyl group (1). Thus, it was not unexpected to find that F values, measured either by the TLC–INH method or a highly specific radioimmunoassay (9), significantly correlated with each other (40 plasma samples, $r = 0.96$; Fenske, unpublished data). Another feature of this method may deserve some attention: Except in two other studies analyzing F excretion in the guinea pig (3,4), the authors used normal TLC plates; that is, plates without concentrating zones. However, our experience from the present and previous work (3,4) is that quantitative estimation of F and/or F-like steroids is best accomplished by the use of TLC plates with concentrating zones because even large volumes of extracts (0.1 mL) could be applied in quite uniform bands on the plates. The sensitivity of the TLC–INH method is approximately 20 ng/mL; this is much poorer than sensitivity reported for other, currently used methods [0.1–0.2 ng/mL (12,13) and 1.0 ng/mL (14)]. However, the relatively low sensitivity of the TLC–INH method is not critical because F concentration in human plasma is well over the range which necessitates operation of the scanner at a lower sensitivity. There are some important advantages of the TLC–INH method: (*i*) it is easier to perform than a corresponding high-performance liquid chromatography method; (*ii*) eight to nine samples can be analyzed on a single plate with three standards, leading to faster analyses and a lower cost of solvent usage and disposal; (*iii*) plasma extracts can be developed in one or more solvent systems, making sure that F is well-separated from other steroids or caffeine (Table I); (*iv*) internal standardization is not necessary because a calibration curve is generated on each plate by processing samples and standards under the same conditions; (*v*) attention to the analyst is not required during development, drying steps, or densitometry; (*vi*) the storage of complete chromatograms on individual plates as analytical discs (15) is possible; and (*vii*) the possibility exists of running several standards or reference lanes simultaneously, also called "in-system calibration" (15).

Conclusion

The present work shows that TLC plates can be used with advantage to measure INH-labeled F. The results presented here also show that the INH method is simple, rapid, specific, sensitive, reproducible, and economical, making this method suitable for clinical and pharmacokinetic studies. This may be especially true for small endocrine research groups having no access to expensive high-performance liquid chromatography systems.

References

1. A. Ercoli, L. de Giuseppe, and P. de Ruggiere. Isonicotinil-idrazoni di chetostersteroidi. *Farmaco. Sci. Tec.* **7:** 170–72 (1952).
2. T.E. Weichselbaum and H.W. Margraf. Determination of Δ^4-3-keto-corticosteroids in human peripheral plasma by the isonicotinic acid hydrazide (INH) reaction. *J. Clin. Endocrinol. Metab.* **17:** 959–65 (1957).
3. M. Fenske. Determination of cortisol in plasma and urine by thin-layer chromatography and fluorescence derivatization with isonicotinic acid hydrazide. *Chromatography* **47:** 695–700 (1998).
4. M. Fenske. Determination of cortisol and cortisone in human morning and overnight urine by thin-layer chromatography and fluorescence derivatisation with isonicotinic acid hydrazide. *Chromatographia* **52:** 810–14 (2000).
5. E.J. Umberger. Isonicotinic acid hydrazide as a reagent for determination of Δ^4-3-ketosteroids. Determination of progesterone and testosterone proprionate in oil solutions. *Anal. Chem.* **27:** 768–73 (1955).
6. L.L. Smith and T. Foell. Differentiation of Δ^4-ketosteroids and $\Delta^{1,4}$-ketosteroids with isonicotinic acid hydrazide. *Anal. Chem.* **31:** 102–105 (1959).
7. J.Y.P. Wu. Collaborative study of an assay for progestational steroids in individual contraceptive tablets. *J. Assoc. Off. Anal. Chem.* **60:** 922–25 (1977).
8. M. Blanco, J. Coello, R. Iturriaga, S. Maspoch, and N. Villegas. Kinetic spectrophotometric determination of hydrocortisone acetate in a pharmaceutical preparation by use of partial least squares regression. *Analyst* **124:** 911–15 (1999).
9. M. Fenske. Role of cortisol in the ACTH-induced suppression of testicular steroidogenesis in guinea pigs. *J. Endocrinol.* **154:** 407–14 (1997).
10. H.J. Ruder, R.L. Guy, and M.B. Lipsett. A radioimmunoassay for cortisol in plasma and urine. *J. Clin. Endocrinol. Metab.* **35**: 219–24 (1972).
11. M. Fenske. Rapid and specific determination of free cortisol in guinea pig urine and faeces by thin-layer chromatography-competitive protein-binding assay. *Chromatographia* **50:** 428–32 (1999).
12. R. Taylor, S.K. Grebe, and R.J. Singh. Quantitative, highly sensitive liquid chromatography-tandem mass spectrometry method for detection of synthetic corticosteroids. *Clin. Chem.* **50:** 2345–52 (2004).
13. A.Y. Yang, L. Sun, D.G. Musson, and J.L. Zhao. Determination of M+4 stable isotope labeled cortisone and cortisol in human plasma by µelution solid-phase extraction and liquid chromatography–tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **20:** 233–40 (2006).
14. M.M. Kushnir, R. Neilson, W.L. Roberts, and I.L. Rockwood. Cortisol and cortisone analysis in serum and plasma by atmospheric pressure photoionization tandem mass spectrometry. *Clin. Biochem.* **37:** 357–62 (2004).
15. B. Renger, H. Jehle, M. Fischer, and W. Funk. Validation of analytical procedures in pharmaceutical analytical chemistry: HPTLC assay of theophylline an an effervescent tablet. *J. Planar Chromatogr.* **8:** 269–78 (1995).
16. R.O. Quesenberry and F. Ungar. Thin-layer chromatographic systems for adrenal corticosteroids. *Anal. Biochem.* **8:** 192–99 (1964).
17. S.M.H. Ak-Habet and H.J. Rogers. Two chromatographic methods for the determination of corticosteroids in human biological fluids: pharmacokinetic applications. *J. Pharmac. Sci.* **78:** 660–66 (1989).

Manuscript received October 25, 2006;
revision received June 27, 2007.