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Journal of Chromatography B, 652 (1994) 83–89

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short Communication

Solvent and solid-phase extraction of natural and synthetic corticoids in human urine

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(First received August 13th, 1993; revised manuscript received November 16th, 1993)

Abstract

Optimization of the main variables that affect solvent and solid-phase extraction processes, using disposable C_{18} cartridges and the non-ionic polymeric resin Serdolit AD-2, of human urine containing natural and synthetic corticoids is described. The data were obtained from different HPLC separations of these compounds using calibration graphs obtained before and after extraction of these compounds. The procedures, including sample preconcentration, showed efficiencies over 90%. NaCl was used to avoid emulsion formation in solvent extraction. The results achieved using solvent and solid-phase extraction are discussed.

1. Introduction

Corticoids (CC) are excreted in urine basically as their conjugated metabolites and in the non-metabolized form [1,2]. These compounds are detected and quantified in biological fluids with different purposes, *e.g.* calculation of pharmacokinetics parameters, development of new corticoids, monitoring of therapeutic doses, and recently in doping control [3–5]. One of the most employed techniques for analyzing corticoids is reversed-phase high-performance liquid chromatography (RP-HPLC).

Usually, preliminary sample preparation is needed due to the occurrence of interfering compounds or the low levels of the analytes in the sample. The majority of the sample preparation procedures uses solvent extraction (SE). The major disadvantage of SE is emulsion formation which causes loss of compounds, leading to lower and variable recoveries. Emulsion formation can be overcome by using larger volumes of the extracting solvent relative to sample size, by using less vigorous mixing methods or by adding reagents to the aqueous phase. An alternative to SE is solid-phase extraction (SPE) with materials such as carbon, celite, Florisil and alumina. Also ion-exchange resin-loaded paper, non-ionic resins such as Amberlite XAD-2, and sorbents on disposable cartridges have been used for the screening of urine samples for drugs [6,7].

In previous papers SE and SPE data of corticoids have been reported [8–15], however, this information is not extensive. This paper describes improved SE and SPE procedures for urine samples containing corticoids by exhaustive

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SSDI 0378-4347(93)E0396-8

optimization of the main extraction variables. Moreover, a comparison between the recoveries obtained with SE and SPE is reported. The extraction data were assessed from different HPLC separations [16]. The addition to urine samples of reagents which can avoid emulsion formation in SE was also studied.

2. Experimental

2.1. Chemicals and reagents

Triamcinolone (TRI) (9α - fluoro - 11β,16α, 17,21 - tetrahydroxy - 1,4 - pregnadiene - 3,20 - dione), prednisone (PS) (17α,21 - dihydroxy - 1, 4 - pregnadiene - 3,11,20 - trione), cortisone (CS) (4 - pregnene - 17α - 21 - diol - 3,11,20 - trione), prednisolone (PL) (1,4 - pregnadiene - 11β,17α,21 - triol - 3,20 - dione), cortisol (CL) (11β,17α,21 - trihydroxypregn - 4 - ene - 3,20 - dione), dexamethasone (DM) (9α - fluoro - 16α - methylprednisolone), betamethasone (BM) (1, 4 - pregnadiene - 3,20 - dione - 9α - fluoro - 16β - methyl - 11β,17α,21 - triol), corticosterone (CT) (4 - pregnene - 11β,21 - diol - 3,20 - dione), 11α - hydroxyprogesterone (HP) (4 - pregnene - 11α - ol - 3,20 - dione), fluoro - cortisone (FL) (9α - fluoro - 11β,17α,21 - trihydroxy - 4 - pregnene - 3,20 - dione), fluorocortisone acetate (FLA) (9α - fluoro - 11β, 17α,21 - trihydroxy - 4 - pregnene - 3,20 - dione acetate), deoxycorticosterone (DC) (4 - pregnen - 21 - ol - 3,20 - dione), methylprednisolone (MPL) (6α - methyl - 11β,17α,21 - trihydroxy - 1,4 - pregnadiene - 3,20 - dione), triamcinolone acetnonide (TRA) (1,4 - pregnadiene - 9α - fluoro - 3,20 - dione - 11β,16α,17α,21 - tetrol - 16,17 acetonide) were purchased from Sigma (St. Louis, MO, USA) and methanolic solution were prepared. All compounds are detectable by HPLC - UV.

HPLC-grade acetonitrile was purchased from Promochem (Wesel, Germany). Millipore 0.45-μm nylon filters (Bedford, MA, USA), C_{18} Extrasep cartridges (2.8 ml and 500 mg) (Phenomenex, Torrance, CA, USA), and phenyl-cyano C_8 Bond Elut (1.0 ml and 100 mg) from Analytichem International (Harbor City, CA, USA), were used. Serdolit AD-2 resin (0.1–0.2 mm) (Serva, Heidelberg, Germany) was used as water-slurry. Other chemicals were of analytical reagent grade.

2.2. Apparatus

The chromatographic system consisted of the following components all from LDC Analytical (Riviera Beach, FL, USA): a Constametric 4100 solvent delivery system, a spectromonitor 5000 photodiode-array detector covering the range 190–360 nm and interfaced to a computer for data acquisition, recorder Model CI 4100 data module. A Rheodyne 20-μl loop injector (Cotati, CA, USA), a Jones-Chromatography block heated series 7960 (Seagate Technology, Scotts Valley, CA, USA) for thermostatting columns at 30°C, and a bonded-silica Hypersil ODS (250 mm × 4.6 mm I.D.) 5 μm column from Phenomenex (Torrance, CA, USA), were used. The vortex-mixer Mixo-Tub-30 was from Crison (Barcelona, Spain) and the Visiprep vacuum manifold system from Supelco (Bellefonte, PA, USA).

2.3. Mobile phase

The mobile phase was prepared by mixing Milli-Q water with acetonitrile at the required volume ratio, programming the pump. Acetonitrile and water were previously filtered under vacuum through 0.45-μm nylon filters and degassed using helium sparge.

2.4. Sample preparation

Natural corticoid-free urine was initially prepared by percolating the urine through a Pasteur pipette (230 mm × 7 mm) containing a 10-mm plug of Serdolit AD-2 resin. The eluates were collected and used as matrix for corticoid spikes.

Other ways to obtain this matrix, such as collection of urine from patients suffering from Addison disease or after administration to a

normal subject of 1 mg dexamethasone [17], provided similar results. For synthetic corticoids, urine samples of normal subjects were used.

2.5. Solvent extraction

Human urine samples (3 ml) containing corticoid concentrations in the range 0.13–0.66 μg/ml were placed in stoppered centrifuge tubes. NaCl (0.35 g) was added and the pH was adjusted to 9.0 with 0.5 g Na_2HPO_4. A volume of 4 ml of dichloromethane was added. The tube was vortex-mixed for 1 min and centrifuged for 3 min at 3700 *g*. The organic phase was removed and dried over anhydrous Na_2SO_4. A 3-ml aliquot was evaporated to dryness under vacuum. The dried residue was reconstituted with 200 μl of 5 μg/ml I.S. (MPL or PS) and 20 μl were injected onto the HPLC system.

2.6. Extraction with C_{18} cartridges

Human urine samples (3 ml) containing corticoid concentrations in the range 0.13–0.66 μg/ml were adjusted to pH 7.5 with NaOH. These samples were processed with a vacuum manifold system through Extra-Sep C_{18} cartridges which previously had been conditioned with 5 ml of methanol followed by two 5-ml volumes of water. The cartridge was washed with 5 ml of water–acetone (4:1, v/v) and 1 ml of *n*-hexane. The elution was accomplished using two 2-ml volumes of diethyl ether. The eluate was processed by SE.

2.7. Extraction with Serdolit AD-2

To 3 ml of human urine sample containing corticoid concentrations in the range 0.13–0.66 μg/ml, 0.5 g Na_2HPO_4 was added to adjust the pH to 9.0. The sample was passed through a Pasteur pipette (230 mm × 7 mm) containing a 10-mm plug of Serdolit AD-2 resin. The plug was rinsed with 2 ml demineralized water and 0.5 ml of *n*-hexane; two 1.0-ml volumes of methanol were added to elute the corticoids. The eluate was processed by SE.

3. Results and discussion

3.1. Preliminary considerations

Urine samples (3 ml) were used in all assays. SE and SPE recoveries, *%E*, for the compounds shown in Table 1 were assessed from different HPLC separations [16]. Chromatograms, compounds and separation conditions are shown in Fig. 1. Two different calibration graphs were prepared using 5 μg/ml of I.S. Peak-area response ratios were plotted against concentration. The first calibration was obtained by direct injection of standard solutions of corticoids with concentrations in the range 2–10 μg/ml. Calibration graphs were linear (correlation coefficients were higher than 0.999) and each slope value, s_1, was calculated. After optimizing the extraction process, a second calibration graph in the range 0.13–0.66 μg/ml was obtained from urine samples containing corticoids after SE or SPE (see procedures) and the slope value, s_2,

Table 1
SE and SPE recoveries, (*%E*) ± R.S.D., for corticoids added to urine samples. Amount added 0.13–0.66 μg/ml

Compound	SE	C_{18} cartridges	Serdolit AD-2
1 (TRI)	91.0 ± 4.3	91.3 ± 3.5	90.5 ± 4.2
2 (PS)	97.0 ± 5.1	94.0 ± 2.3	106 ± 2.5
3 (CS)	108 ± 5.6	98.2 ± 1.6	95.6 ± 3.9
4 (PL)	95.8 ± 5.0	94.2 ± 5.0	105 ± 4.1
5 (CL)	98.7 ± 0.5	97.3 ± 4.1	101 ± 2.2
6 (FL)	91.7 ± 2.6	90.2 ± 5.3	107 ± 3.7
7 (CT)	94.9 ± 3.6	94.0 ± 1.6	96.1 ± 3.0
8 (BM)	102 ± 2.3	101 ± 0.3	100 ± 2.6
9 (DM)	94.4 ± 3.4	93.0 ± 6.6	92.7 ± 2.8
10 (MPL)	94.4 ± 5.2	93.0 ± 5.6	101 ± 3.7
11 (FLA)	91.0 ± 6.5	104 ± 3.6	94.3 ± 3.1
12 (TRA)	97.0 ± 4.4	107 ± 6.5	93.0 ± 4.0
13 (HP)	104 ± 6.0	98.6 ± 5.8	105 ± 3.1
14 (DC)	99.1 ± 9.5	90.1 ± 7.6	97 ± 4.2

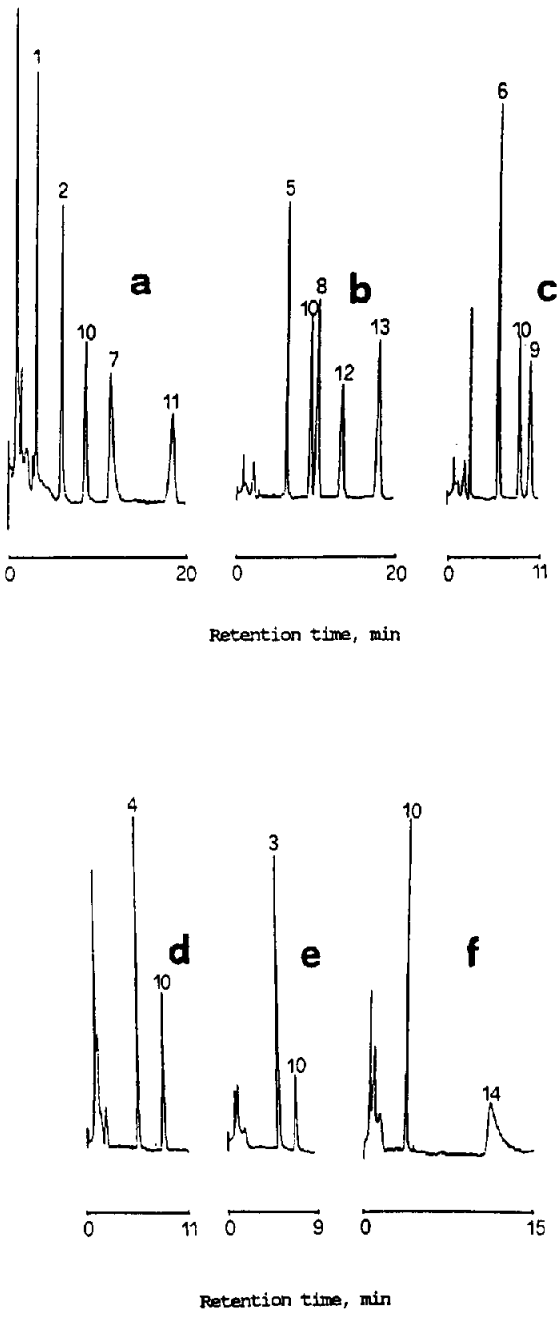

Fig. 1. HPLC of a standard mixture containing corticosteroids (10 μg/ml). Conditions: water–acetonitrile as mobile phase (68:32, v/v) for (a), (b), (c), (d) and (e), and (60:40, v/v) for (f) chromatograms, Hypersil 5-ODS (250 mm × 4.6 mm I.D.) column (30°C), flow-rate, 1.0 ml/min; UV detection at 245 nm. Injection volume: 20 μl. Peak numbers as in Table 1.

was calculated. The $\%E$ values were calculated using the equation $E(\%) = (s_2/s_1) \cdot 100$.

3.2. Optimization of solvent extraction

The most useful solvents in SE of urine samples containing corticoids are dichloromethane, diethyl ether and ethyl acetate [18]. A preliminary study was carried out as follows: Na_2HPO_4 (0.5 g) was added to samples; extraction ratio, $r = V_o/V_w = 1$ (ratio between the volumes of the organic and aqueous phase); 5 min agitation time. The recoveries obtained were: 79–82% with diethyl ether, 85–95% with dichloromethane, and 87–92% with ethyl acetate. Dichloromethane was finally selected.

The influence of the extraction ratio, r, on the recovery of corticoids was studied in the range 1–1.7 with dichloromethane. An r value of 4/3 was finally chosen because at such a ratio maximum extraction efficiency was reached. Several examples are shown in Fig. 2.

The effect of agitation time on the extraction was studied. The samples were vortex-mixed for 0.5–5.0 min. Optimum recovery of corticoids was reached at 1.0 min. For further experiments this agitation time was selected.

The effect of pH was studied by addition of sodium acetate, Na_2CO_3, NaOH or Na_2HPO_4 to urine samples. After adding each solid compound (to a final concentration of 2.5 mM), the pH was measured (range 4.7–9.0). As expected, no significant effect of pH on recovery was observed. Na_2HPO_4 was selected (pH 9) for the HPLC determination of corticoids in urine samples. Thus, ionization of the compounds present in urine is favored and, moreover, the further chromatographic determination is improved. Chromatograms at different pH values are shown in Fig. 3.

The effect of ionic strength was studied with Na_2HPO_4 or K_2HPO_4 over the range 2.5–8.7 mM. The extraction increased slightly with increasing Na_2HPO_4 concentration and no significant differences were observed between the salts used. The optimum concentration of Na_2HPO_4 was 6.22 mM (0.5 g was added to urine samples without taking into account volume effects).

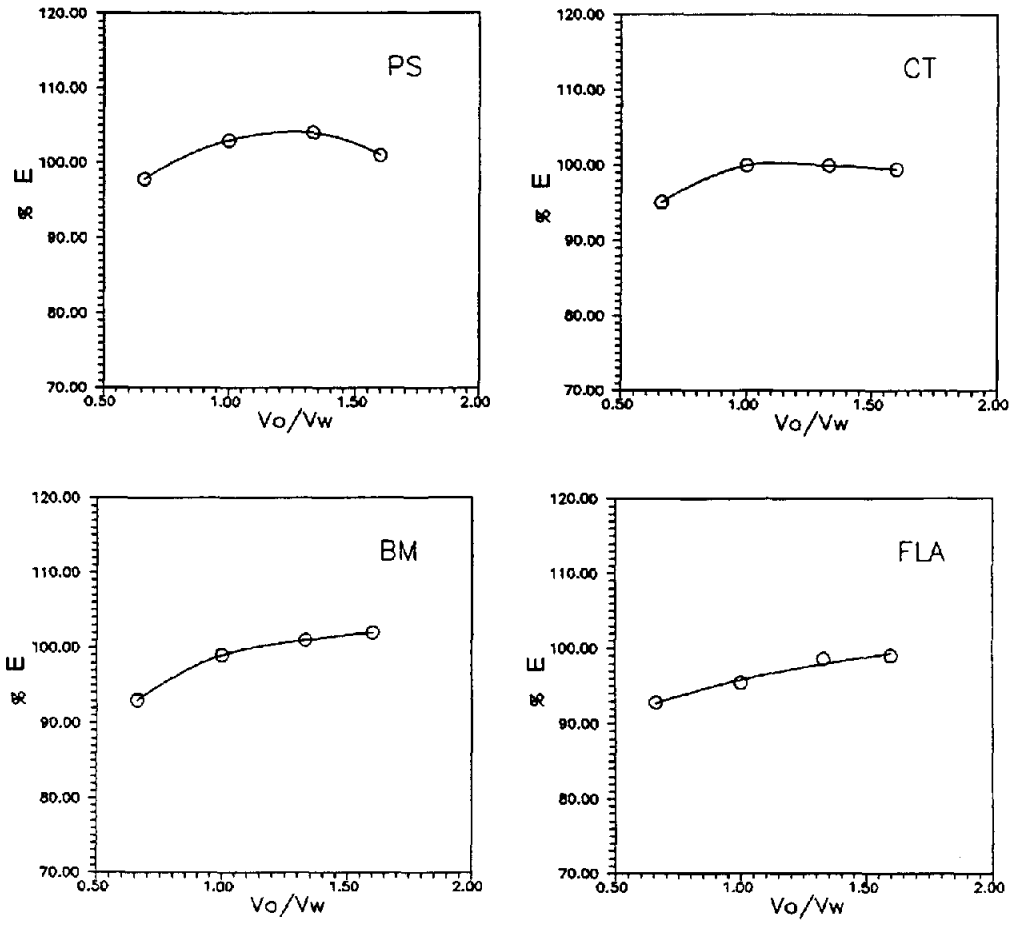

Fig. 2. Effect of extraction ratio ($r = V_o/V_w$), on the extraction efficiency in SE for PS, CT, BM and FLA.

Addition of NaCl (0.35 g) to urine samples inhibited emulsion formation and has no significant effect on recovery.

3.3. Optimization of SPE extraction

SPE with C_{18} cartridges and Serdolit AD-2 were studied. The chromatographic retention of corticoids using HPLC-C_{18} columns is independent of pH. Sample preparation was carried out by adjusting the pH to 7.5 with NaOH or 50 m*M* phosphate buffer. Urine samples were passed under vacuum through the cartridges previously conditioned (see procedure) and washing was accomplished with 5 ml of a mixture of water–acetone (4:1, v/v), 1 ml of *n*-hexane and 2 ml of water, without significant loss of the analytes. Elution was studied by using ethyl acetate, dichloromethane, methanol and diethyl ether. Two volumes of 2 ml of diethyl ether were chosen. The eluates were processed according to the SE procedure. The results are shown in Table 1. Similar results were obtained with C_8. SPE with Serdolit AD-2 resin was carried out as described in the Experimental section. The results are shown in Table 1.

3.4. Precision and accuracy

The precision was evaluated by analyzing four different samples of corticoids containing 5 μg/ml each. The relative standard deviation (R.S.D.) for each corticoid is shown in Table 1. The accuracy was assessed in the 2–10 μg/ml

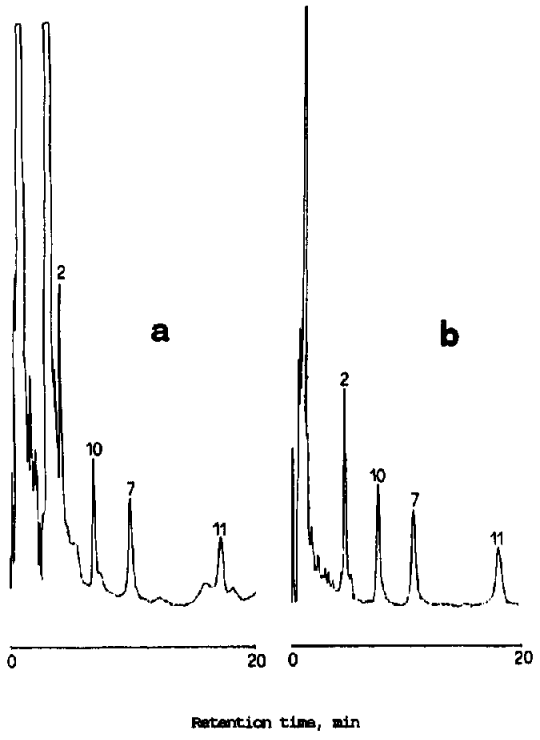

Fig. 3. Chromatograms obtained at different extraction pH values in SE: (a) 2.5 m*M* acetate buffer pH = 4.5, and (b) 6.22 m*M* phosphate buffer (pH = 9). Peak numbers as in Table 1.

range for each corticoid by applying the Barlett and Harley test [19]. The results confirmed that the developed methods do not present a systematic error and do not require a blank correction.

4. Conclusions

The aim of this work was to compare SE and SPE for urine samples spiked with corticoids (Table 1). These processes will serve as a starting point in the quantification of the corticoids shown in Table 1 which might be found in urine. In order to increase the detection for any glucocorticoid, hydrolysis of the glucuronides with β-glucuronidase is recommended. Assays for actual steroids in human urine will require further assessment of sensitivity and specificity, since there are numerous metabolites that may interfere in the HPLC separation [20]. In this paper some progress has been made. A study involving 14 compounds and 3 different extraction procedures, including new extraction data, is reported. Recoveries have been improved and showed to be independent of concentration. In SE, emulsion formation was avoided. Comparing SE and SFE, the best recoveries have been achieved using Serdolit AD-2 giving an acceptable clean-up of the samples. Using disposable C_8 and C_{18} cartridges, the time necessary for analysis was the lowest and the resulting chromatograms were very clean. A good time/cost ratio was achieved using Serdolit AD-2 which gives the best efficiency data and acceptable clean-up of the samples.

5. Acknowledgement

This work was supported by the Consejo Superior de Deportes and CICYT grant DEP. 320/91.

6. References

[1] R.H. Williams, J.D. Wilson and D.W. Foster (Editors), *Williams Textbook of Endocrinology*, W.B. Saunders Company, Philadelphia, 7th ed; 1985.
[2] P. Métais, J. Agneray, G. Férard and J.C. Fruchart, *Biochimie Clinique*, Simep, Paris, 1988.
[3] S.J. Park, Y.J. Kun and H.S. Pyo, *J. Anal. Toxicol.*, 14 (1990) 102.
[4] H. Derendorf, P. Rohdewald, G. Hochnans and H. Möllmann, *J. Pharm. Biomed. Anal.*, 4 (1986) 197.
[5] M.H. Sayed, Al-Habet and M.J. Rogers, *J. Pharm. Sci.*, 78 (1989) 660.
[6] R.D. McDowall, J.C. Pearce and G.S. Murkitt, *J. Pharm. Biomed. Anal.*, 4 (1986) 3.
[7] V.K. Prasad, B. Ho and C. Haneke, *J. Chromatogr.*, 378 (1986) 305.
[8] Z. Saito, E. Amatsu, T. Ono, S. Himumi, T. Mimol, T. Hashiba, S. Sakato, M. Miyamoto and R. Takeda, *Folia Endocrin. Japan.*, 55 (1979) 1296.
[9] T. Seki and Y. Yamaguchi, *J. Liq. Chromatogr.*, 6 (1983) 1131.
[10] M. Yamaguchi, T. Yositake, J. Ishida and M. Nakamura, *Chem. Pharm. Bull.*, 37 (1989) 3022.
[11] M. Takeda, M. Maeda and A. Tsujy, *Biomed. Chromatogr.*, 4 (1990) 119.
[12] A. Sudo, *J. Chromatogr.*, 528 (1990) 453.

[13] J.A. Shah and D.J. Weber, *J. Chromatogr.*, 496 (1989) 245.
[14] M. Schoneshofer, A. Fenner, G. Altinok and H.J. Dulce, *Clin. Chim. Acta*, 106 (1980) 63.
[15] P. Gaignage, G. Lognay, M. Marlier, M. Severin and P. Dreze, *Chromatographia*, 26 (1989) 12.
[16] A. Santos-Montes, A.I. Gasco-López and R. Izquierdo-Hornillos, *J. Chromatogr.*, 620 (1993) 15.
[17] G.W. Liddle, *J. Clin. Endocrinol.*, 20 (1960) 1539.
[18] J.H.M. Van der Berg, C.H.R. Mol, R.S. Deelder and J.H.M. Thyssen, *Clin. Chim. Acta*, 78 (1977) 165.
[19] L. Sachs, *Applied Statistics. A Handbook of Technique*, Springer Verlag, Berlin, 1978, p. 500.
[20] V. Garg and W.J. Jusko, *J. Chromatogr.*, 567 (1991) 39.