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DETERMINATION OF URINARY CORTISOL WITH THREE COMMERCIAL IMMUNOASSAYS

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Summary—Urinary cortisol determination was performed with three commercially available immunoassays: one enzyme-immunoassay (Cortisol Biotrol) (EIA) and two radioimmunoassays: Quanticoat Cortisol (Kallestad Diagnostics) (KD-RIA) and GammaCoat Cortisol (Clinical Assays) (CA-RIA). Four procedures were carried out. Procedure I (methylene chloride extraction) was applied to EIA and CA-RIA and procedure II (ethyl acetate extraction) to KD-RIA. Procedure III combining procedure I and column chromatography on Sephadex LH 20 in methylene chloride was applied to the three kits. Procedure IV consisting of carbon tetrachloride preextraction and extraction with cyclohexane–ethyl acetate (50:50, v/v) was applied to CA-RIA. The results obtained were compared with those of the reference technique, "on-line" HPLC with u.v. detection. Two groups of results were arbitrarily considered, those below ($n = 28$) and those above ($n = 6$) 270 nmol/l. In the first group, the results were markedly overestimated when the procedure was limited to solvent extraction. Conversely, the third procedure proved the efficiency of the chromatographic step since specificity was greatly improved in the three cases, the levels obtained with either kits being similar to those of the reference technique. The second group of results (above 270 nmol/l) yielded by the three kits were not always higher than those of HPLC when the procedure was limited to solvent extraction. When column chromatography was included in the procedure, the results were comparable to those of HPLC in three cases and lower in the three others. Since, the latter samples were collected after cortisol administration, and overestimated cortisol values obtained by HPLC might be due to the interference of some cortisol metabolites.

INTRODUCTION

Since urinary unconjugated cortisol has been shown to reflect the non-protein-bound serum fraction of the circulating hormone [1] and the cortisol production rate [2, 3], its determination has been considered as the screening test of choice for Cushing's syndrome [4]. Thus, many techniques have been devised for this purpose yet radioimmunoassay (RIA) remains the most commonly used though HPLC methods have become more and more widely applied since the early 1980s [5–13]. In fact, they were shown to be more specific than RIA whether they were followed by RIA [5] or by u.v. detection [7–9].

More than a decade ago, alternatives to RIA were proposed [cf. review 14] to overcome the drawbacks of radioactivity and the problems related to its manipulation in clinical laboratories. Among these methods enzyme-immunoassays (EIA) were the most widely used and whatever the nature of the labelling enzyme used was they have been shown to be convenient for cortisol evaluation in plasma [15–19]. In fact the results obtained with these methods were similar to those yielded by RIA. Nowadays, many of these methods are commercially available as ready-to-use kits and some of them have also been proposed for urinary cortisol determination. To our knowledge, only one study has appeared in the literature concerning urinary cortisol determination with ELISA (enzyme-linked immunosorbent assay) [20]. The aim of this paper was to study one EIA and two RIA kits and to compare the results obtained with those of a specific HPLC [13]. For this comparison, different procedures consisting of solvent extraction only or followed by column chromatography on Sephadex LH 20 as previously described for routine urinary and plasma cortisol determination by RIA [21, 22] were performed.

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MATERIALS AND METHODS

Urine samples

Urine specimens collected by 34 hospitalized patients were stored at −20°C until assayed.

EIA

The kit studied was designed for serum cortisol determination (Cortisol Lab Biotrol, Paris) (EIA). Each serum aliquot (20 μl) was incubated simultaneously with cortisol conjugated to alkaline phosphatase and a bead coated with monoclonal antibody anti-cortisol. After incubation, the beads are washed to remove unbound materials then incubated with phenyl phosphate and amino-4-antipyrine. The liberated phenol develops a red orange colouration in the presence of amino-4-antipyrine and potassium ferricyanide. The intensity of the colour measured spectrophotometrically at 492 nm is proportional to the amount of cortisol present in the sample.

According to Table 1 where the cross-reactions of different steroids are grouped, as reported by the manufacturer, the assay appears to be specific only when neither exogenous corticosteroids nor metopirone are administered.

RIA

The two tested kits use antiserum-coated tubes and may be applied for cortisol determination either in serum or in urine.

In the first RIA kit, Quanticoat-Cortisol (Kallestad Diagnostics) (Diagnostics Pasteur, Marnes la Coquette) (KD-RIA), the antiserum is polyclonal and cross-reacts significantly with prednisolone, 21-deoxycortisol, 11-deoxycortisol, deoxycorticosterone and 6β-hydroxycortisol (Table 1).

Determination of urinary cortisol may be performed either directly or after extraction with ethyl acetate.

Table 1. Cross-reactivity (%) of the cortisol antisera of the three tested kits as reported by the manufacturers

Steroid	EIA	KD-RIA	CA-RIA
Cortisol	100	100	100
Prednisolone	25.0	45.0	77.0
21-Deoxycortisol	—	14.2	—
Deoxycorticosterone	—	13.1	0.1
11-Deoxycortisol	1.6	4.2	6.3
6β-Hydroxycortisol	—	1.2	—
Corticosterone	2.9	0.4	<0.1
Prednisone	7.0	0.4	0.2
Dexamethasone	0.01	0.14	0.2
17-Hydroxyprogesterone	0.06	0.12	1.2
Tetrahydrocortisone	—	0.05	0.1
Cortisone	17.0	0.01	<0.1
Methylprednisolone	—	—	43.0

In the second kit, GammaCoat Cortisol (Clinical Assays) (Incstar) (Sorin France, Antony), (CA-RIA), the antiserum is also polyclonal and has significant cross-reactions with prednisolone, 6-methylprednisolone, 11-deoxycortisol and 17-hydroxyprogesterone (Table 1).

Urinary cortisol may be determined either directly on a urine aliquot or after extraction with methylene chloride. In addition, a third protocol may be applied and consists of a preextraction step with carbon tetrachloride followed by reextraction of the aqueous phase with the solvent mixture, ethyl acetate–cyclohexane (50:50, v/v).

Determination of urinary cortisol

Urinary cortisol was determined either after solvent extraction with methylene chloride (procedure I) or ethyl acetate (procedure II) or after methylene chloride extraction and column chromatography on Sephadex LH 20 in the conditions described previously [22] (procedure III). In addition, cortisol was also determined by CA-RIA according to procedure IV (see below) as recommended by the manufacturer.

Extraction with methylene chloride (procedure I). In procedure I, applied with EIA and CA-RIA kits, urine aliquots made up to 1 ml with bidistilled water were extracted with 6 ml methylene chloride. The solvent phase was separated and aliquots of 2 and 1 ml were pipetted and evaporated to dryness under a stream of nitrogen. The residues were redissolved with either 200 μl of the dilution buffer of Biotrol kit (EIA) or 200 μl of ethanol. In the case of EIA, an aliquot of 20 μl of the dilution buffer was taken out and submitted to the procedure designed for plasma cortisol determination. In the second case (CA-RIA), an aliquot of 50 μl of ethanol was taken out to the coated tube and evaporated to dryness. The residue was redissolved with 10 μl of cortisol serum blank. After addition of the tracer, the tubes were incubated at 37°C for 45 min then decanted and counted.

Extraction with ethyl acetate (procedure II). This extraction procedure was performed with the KD-RIA kit. Urine aliquots made up to 0.2 ml with bidistilled water were extracted with 2 ml ethyl acetate. The solvent phase was separated and evaporated to dryness. The residue was redissolved with 200 μl ethanol. An aliquot of 50 μl was pipetted in the coated tube and evaporated to dryness. After addition of 20 μl of the zero calibration standard and I^{125}-labelled

tracer, the tubes were incubated at 37°C for 30 min then decanted and counted.

Extraction with methylene chloride followed by column chromatography (procedure III). This procedure was applied with the three kits. The methylene chloride extraction was carried out as described above except that approx. 4000 cpm of tritiated cortisol were added to urine aliquots to monitor methodological losses. The dry residue obtained after evaporation of the whole urine extract was redissolved with 2 × 0.2 ml methylene chloride and applied to Sephadex LH 20 column (i.d: 8.5 mm; height: 170 mm; 10 ml disposable pipettes) prepared in the same solvent [22]. The first eluate consisting of 32 ml methylene chloride was discarded. The second 11 ml fraction eluting cortisol was collected and evaporated to dryness. The residue was redissolved with 6 ml of methylene chloride. The rest of the procedure was as described above.

Preextraction with carbon tetrachloride and extraction with ethyl acetate–cyclohexane (procedure IV). This procedure was applied with the CA-RIA kit only. Urine aliquots made up to 0.3 ml with bidistilled water were extracted with 3 ml carbon tetrachloride. The solvent phase was discarded and the aqueous phase re-extracted with 5 ml of ethyl acetate–cyclohexane (50:50, v/v). The solvent phase was decanted and evaporated to dryness. The residue was redissolved with 6 ml of methylene chloride. The next steps were similar to those described above.

HPLC

The method for the specific determination of non-conjugated cortisol by HPLC has already been described and evaluated [13]. The procedure used a sample cleanup step to remove non-specific u.v.-absorbing compounds. To a urine aliquot of 0.05 to 1.0 ml dexamethasone was added as internal standard. The urine sample was then poured on a reversed-phase column: Bond Elut TM C18 (Analytichem International-Prolabo, Paris, France). The bulk of impurities was removed with alkaline, acid and organic washes. After selective elution with ethanol, steroids were separated on a Lichrosorb diol HPLC column with detection of absorbance at 254 nm. Intra-assay variability was 6.41% at the mean level of 268 nmol/l and inter-assay variability was 14.42% at the mean level of 1392 nmol/l.

Statistical studies

The regression lines were calculated by the least-squares method, with results by HPLC method as the independent variable. The statistical significance of the slopes and the intercepts with the ordinate axis was calculated by Student's t-test.

RESULTS

Validation of the Kits

Accuracy was assessed by adding increasing amounts of unlabelled cortisol to aliquots of a 24-h urine collected after dexamethasone treatment. For each quantity, varying between 690 and 43 pmol/ml, determinations were performed in triplicate. The equation of the regression line calculated between the amounts measured (Y) and those added (X) was performed for each kit and with each of the procedures described above. The results are grouped in Table 2.

When the procedure was limited to solvent extraction the results differed according to the considered kit. Concerning EIA and KD-RIA, though the methodological losses were not corrected, the results were overestimated, the slope being significantly higher than 1 and the intercept with the ordinate axis different from 0. In the case of CA-RIA, the slope of the equation of the two lines corresponding to the two extraction procedures were lower than 1 because methodological losses were not corrected. However, the intercept with the ordinate axis was significantly different from 0 only when extraction was performed with methylene chloride (Table 2).

Table 2. Accuracy of the three kits applied according to the different procedures (urine collected after dexamethasone therapy spiked with increasing amounts of cortisol varying between 43 and 690 pmol/ml)

	Kit		
Procedure	EIA-B	KD-RIA	CA-RIA
I	$Y = 1.24X + 25.03$	—	$Y = 0.91X + 5.82$
II	—	$Y = 1.14X - 22.28$	—
III	$Y = 1.01X - 0.04$	$Y = 1.02X + 0.02$	$Y = 1.02X - 2.93$
IV	—	—	$Y = 0.83X + 3.15$

For a description of the procedures see the text.

Satisfactory results were obtained with the inclusion of a column chromatography step in the procedure. In fact, in the equation of the regression lines calculated for the three kits, the slope and the intercept with the ordinate axis were not significantly different from 1 and 0, respectively.

These results were confirmed by those obtained when aliquots of different volumes of the same urine samples were processed through the different procedures performed with the three tested kits. In fact, the intercept with the ordinate axis was not significantly different from 0 when urine extracts were purified by column chromatography prior to the immunoassay. When the procedure was limited to solvent extraction, this intercept was generally different from 0.

Comparison Between the Three Tested Kits and HPLC

Levels below 270 nmol/l

Taking into consideration the levels obtained by HPLC, the data were subdivided into two groups, above ($n = 6$) or below ($n = 28$) 270 nmol/l. The values below 270 nmol/l were used to calculate the equation of the regression lines between the results of HPLC and those of the three kits according to the different procedures.

Comparison between EIA (Biotrol) and HPLC. When cortisol was evaluated in crude urine extracts (procedure I) with the Biotrol kit (EIA), the levels observed were significantly higher than those found with HPLC (Fig. 1). They were overestimated about 120% on average.

However, chromatographic purification of the urine extract (procedure III) has yielded markedly lower levels comparable to those of HPLC. The calculated regression equation was (Fig. 1):

$$Y(\text{EIA}) = (1.024 \pm 0.043)X(\text{HPLC}) + (1.238 \pm 4.582).$$

The intercept with the ordinate axis was not different from 0 but the slope was not significantly different from 1.

Comparison between KD-RIA (Kallestad, Pasteur) and HPLC. Very high levels were obtained when the reagents of the Pasteur kit were applied on crude urine extracts (procedure II). In fact, the levels were on average 2.5 times those of HPLC and in addition, an important systematic error could be evidenced.

These discrepancies between the two techniques disappeared when urine extracts were purified by chromatography on Sephadex LH

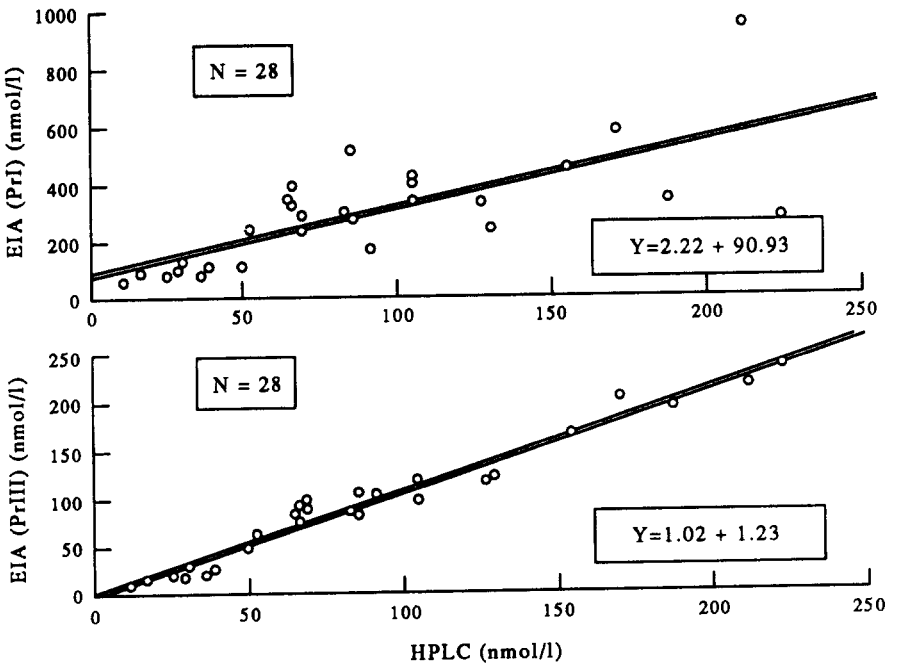

Fig. 1. Correlation between urinary cortisol levels (nmol/l) as measured by HPLC and EIA after procedure I (PrI) (upper figure) or procedure III (PrIII) (lower figure).

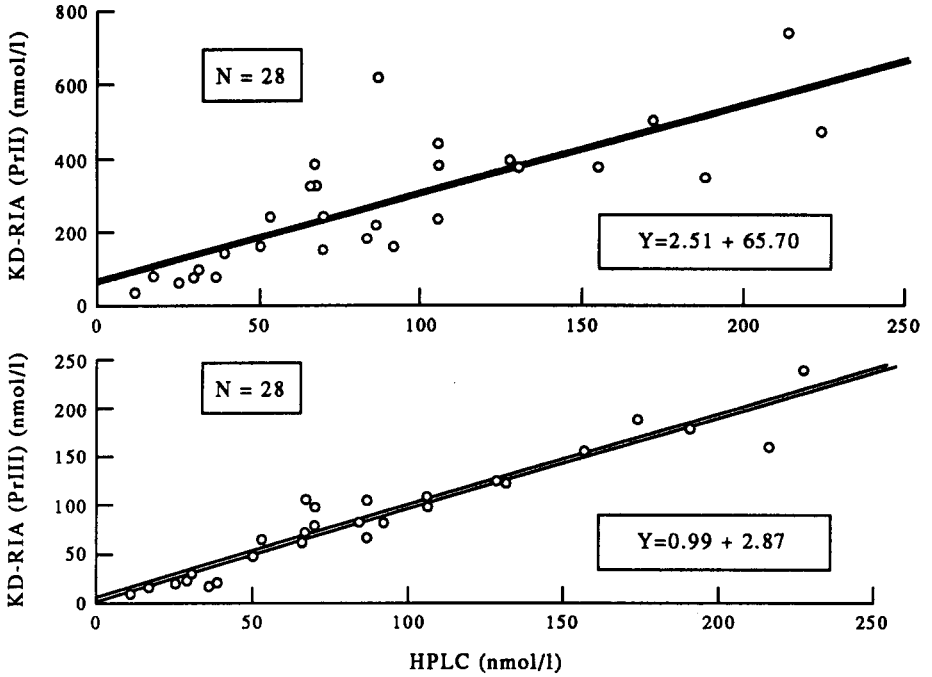

Fig. 2. Correlation between urinary cortisol levels (nmol/l) as measured by HPLC and KD-RIA after procedure II (PrII) (upper figure) or after procedure III (PrIII) (lower figure).

20 column (procedure III). Indeed, the regression equation was (Fig. 2):

$$Y(\text{KD-RIA}) = (0.995 \pm 0.056)X(\text{HPLC}) + (2.877 \pm 5.947).$$

The slope and the intercept with the ordinate axis were not significantly different from 1 and 0, respectively.

Comparison between CA-RIA (Clinical Assays, Sorin) and HPLC. This kit yielded overestimated results when the RIA was performed on either solvent extracts (procedure I or IV) (Fig. 3). However, purification of the extracts with column chromatography (procedure III) has yielded, as with the other kits, markedly lower levels which were comparable to those observed with HPLC. The equation of the regression line was:

$$Y(\text{CA-RIA}) = (0.977 \pm 0.047)X(\text{HPLC}) + (4.622 \pm 4.970).$$

The slope and the intercept with the ordinate axis were not significantly different from 1 and 0, respectively.

Levels above 270 nmol/l

The levels above 270 nmol/l obtained by HPLC and by the three kits tested according to the different procedures described above are reported in Table 3.

Procedures limited to solvent extraction did not always lead to an overestimation of cortisol values as it was observed in the results below 270 nmol/l. Moreover, while in three cases, the purification of the urinary extract by column chromatography has yielded results comparable

Table 3. Urinary cortisol levels above 270 nmol/l obtained by HPLC compared with those observed with the three kits applied according to different procedures

	Urine (nmol/l)					
	1	2	3	4	5	6
HPLC	519	359	400	806	477	290
EIA						
Procedure I	1217	243	483	2571	3606	850
Procedure III	522	168	201	775	428	251
KD-RIA						
Procedure II	2089	339	505	4139	795	403
Procedure III	510	229	212	582	447	243
CA-RIA						
Procedure I	739	265	279	1702	602	386
Procedure III	535	221	199	643	433	257
Procedure IV	803	265	287	1959	665	290

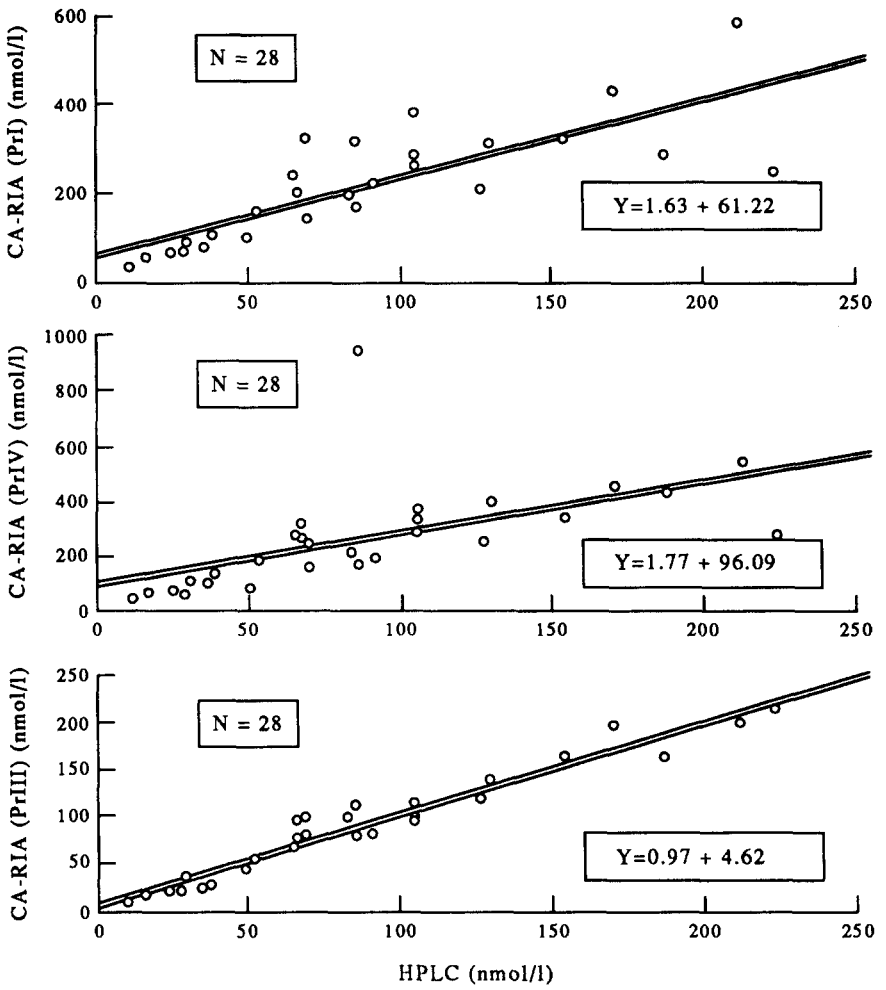

Fig. 3. Correlation between urinary cortisol levels (nmol/l) as measured by HPLC and CA-RIA after procedure I (PrI) (upper figure) or procedure III (PrIII) (lower figure) or procedure IV (PrIV) (middle figure).

to those observed with HPLC, in the three remaining cases (Table 3, samples 2, 3 and 4), the results obtained after Sephadex LH 20 column chromatography with the three kits were markedly lower than those with HPLC. It is noteworthy that the three last urine samples were collected from patients treated with cortisol.

Thus, though these data are limited to draw any conclusion, it might be suggested that determination of urinary cortisol by HPLC may produce falsely high values because of interfering compounds appearing in urine after cortisol administration.

DISCUSSION

In blood plasma, cortisol being quantitatively the most important corticosteroid its determination by competitive protein-binding, RIA, EIA or ELISA may be performed either directly or on crude extracts despite the limited specificity of some of these assays [23].

Concerning urinary cortisol, its determination by competitive protein binding assay (CPB) or by RIA in crude extracts yields overestimated results when compared to those obtained by specific techniques [24–26]. Indeed, many steroidal as well as non-steroidal substances present in urine [2] interfere with these assays and neither solvent extraction nor an acid or alkali wash of the urine extract appeared sufficient to remove them completely [24–29]. In fact, cortisol immunoreactivity only amounted to 51% of the total immunoreactivity of the extract [5]. Thus solvent extraction of urine aliquot followed by chromatographic purification of the extract appears to be mandatory for the reliable determination of urinary cortisol by RIA [2, 28–30]. These two steps remove not only

conjugated cortisol metabolites and the most cross-reactant steroids namely cortisone, 11-deoxycortisol, 21-deoxycortisol and 17-hydroxyprogesterone which appear unconjugated in negligible amounts in urine but also non-steroidal compounds.

Thus it can be anticipated that RIA kits devised for urinary cortisol determination on crude urine extracts would yield overestimated results in comparison with HPLC. This was clearly demonstrated by the present data which confirm the results obtained by Lantto [8] and by Huang and Zweig with KD-RIA [26]. Moreover, the present data have also shown that the levels observed after ethyl acetate extraction (KD-RIA) were the highest in comparison with the two other solvent extraction procedures. In fact, this solvent extracts very polar steroid metabolites particularly 6β-hydroxycortisol which is excreted at higher levels than cortisol in urine [21].

Since the specificity of EIA is comparable to that of RIA it can also be expected that the results obtained with EIA on crude urine extracts would not be reliable in comparison with specific techniques and this was clearly demonstrated by the present data. It should be noted that the data of Lewis *et al.* [20] showing comparable results between EIA and RIA were obtained with direct techniques not including solvent extraction.

The problem of specificity is particularly important and though the cross-reactions, reported by the manufacturers for the three antisera, were generally satisfactory, the levels obtained were markedly overestimated when urine extracts were not purified. This was true whatever solvent was used. Moreover, pre-extraction of urine aliquot with carbon tetrachloride did not greatly improve the specificity of the Clinical Assays kit.

However, the introduction of a column chromatography on Sephadex LH 20 in the procedure has led to results similar to those obtained by HPLC. Yet not any solvent system may be used. In fact, the data of Lewbart and Elverson [9] has clearly shown that the results obtained with RIA (Cortisol-Radioimmunoassay PAK, NEN, MA 02118) following preliminary purification on Sephadex LH 20 column were on the average three times higher than those obtained with TLC–HPLC. Conversely, chromatography on Sephadex LH 20 columns in the conditions already described in detail [22] has proved to be efficient in removing interfering compounds in the final assay whether it was RIA or EIA since the results obtained were comparable to those of HPLC.

In the case of the high levels, above 270 nmol/l, our findings showing higher cortisol levels with HPLC than those observed after column chromatography with the three tested kits when urine samples were collected after cortisol administration are rather difficult to explain. It might be assumed that interfering compounds appearing in urine in these circumstances would yield overestimated cortisol values by HPLC. It might be suggested that these compounds would be cortisol metabolites and the two unconjugated 20α- and 20β-dihydrocortisols [28], being excreted in high amounts in this case, might be responsible for this interference. In any case, Lantto [8] has already observed in some urines falsely higher cortisol levels by HPLC than by isotope dilution-mass spectrometry which is considered as the reference technique. Similarly, Nakamura and Yakata [7] have reported that, in two normal subjects, there was interference with the cortisol HPLC peak by other substances. However, in neither of these two studies was the overestimation of cortisol values by HPLC related to cortisol administration.

In conclusion, it may be suggested that either EIA or RIA may be used for urinary cortisol determination, provided that an adequate chromatographic step is included in the procedure. Among the available chromatographic systems, column chromatography appears to be the most convenient for clinical routine analysis and Sephadex LH 20 in methylene chloride has been shown to be the most adequate for this purpose.

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