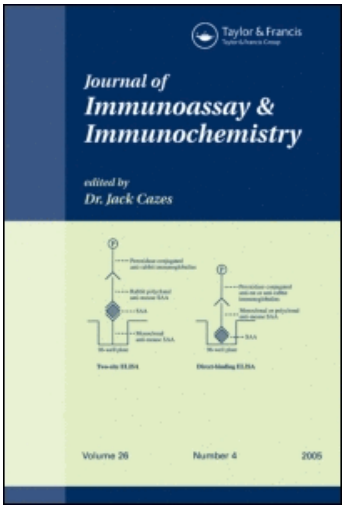

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PITFALLS IN THE ASSAY OF CORTISOL

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ABSTRACT

Many currently used cortisol assays, including commercially available kits, are validated only for adult serum and may give misleading results when applied to urine, fetal or neonatal serum or amniotic fluid. Evidence for such errors is presented to illustrate the danger and confusion which may result. (KEY WORDS: cortisol, cortisone, radioimmunoassay, radiotransinassay, enzyme-linked immunoassay)

INTRODUCTION

Although cortisol was the first non-peptide hormone to be assayed by competitive protein-binding as long ago as 1963 (1), gross errors in its measurement continue to be made and some of these are clinically hazardous. They stem largely from the current reliance on specificity data obtained for a limited number of steroids and the application of methods validated for adult non-pregnant human serum to other types of sample such as urine, amniotic fluid or neonatal serum. Since such errors

occur not infrequently it must be emphasized that each assay must be validated for the tissue, species and circumstances to which it is to be applied. While this is true for all assays, cortisol provides a particularly pertinent example.

Competitive binding assays in current use for cortisol include radiotransinassays (RTA), radioimmunoassays (RIA), and more recently enzyme-linked immunoassays (EIA). All of these rely primarily on the binding-protein for specificity. In RTA, the specificity depends on the species of transcortin (CBG) used and is essentially invariant for all individuals of the same species. In the immunoassays, specificity depends on the antibody and varies according to the site of conjugation used to form the antigen and to the particular batch of antibody obtained, differing to some extent from animal to animal and from time to time in the same animal.

While commercial firms producing antisera to cortisol assess cross reactivity using from 6 to 20 of the most frequent steroids in adult human blood, there are other steroids which occur in relatively greater amounts in urine. Still others are prominent only in sera of subjects with congenital adrenal hyperplasia and some are seen only during pregnancy or fetal life. New steroids continue to be identified, e.g. the cortoic acids (2) and the highly polar steroids comprising the bulk of

cortisol metabolites in neonate urine (3). To date satisfactory reference methods have been lacking; although GCMS appears to be promising.

Other factors which may influence specificity include solvents used for extraction, selective adsorption to agents used to separate bound and unbound fractions of tracer, and additional steps, such as chromatography. Changes in reagents or techniques should be checked using appropriate and relevant control samples.

Urinary Cortisol

Values for urinary cortisol (unconjugated or 'free' cortisol) of about 40 µg/day (range 0 - 120 µg/day) in normal adult subjects were first established in the late 1950's using the highly specific method of double isotope dilution with derivative formation (DIDA), (Table 1). When we first applied the RTA to urine using human CBG in 1968 (4), we obtained closely similar values (mean 44 µg/day, range 0 - 120 µg/day), as did Beardwell et al (5). Although we did not attempt to define a lower limit of normal, later authors did establish a lower limit of 16 µg/day (6). This assay proved to be clinically reliable for the assessment of adrenal function (4,5,6), and in the absence of suitable chromatographic methods for RTA (high blank values were a problem until 1971), we

TABLE 1

URINARY CORTISOL
Mean ± SEM - µg/day

Authors	Ref.	Year	Subjects	Cortisol	Purification	Assay
Ayres et al	(11)	1957	24 men	35 ± ?	ext chrom	DIDA
DeMoor et al	(12)	1962	419 adults	191 ± ?	wash + ext	Fluometric
Murphy	(4)	1968	48 adults	48 ± 6	ext	RTA (human)
Beardwell et al	(5)	1968	24 adults	42 ± 7	ext	RIA (human)
Hsu & Bledsoe	(6)	1970	34 adults	33 ± ?	wash + ext	RTA (human)
Ruder et al	(7)	1972	8 adults	43 ± 9	ext + chrom	RIA
Apter et al	(8)	1975	9 women	60 ± 23	ext + chrom*	RIA
Chattoraj et al	(9)	1976	10 women	18 ± 2	ext + chrom	RIA
Stahl et al	(10)	1976	42 adults	36 ± 3	ext	RTA (horse)
			"	43 ± 3	ext	RIA
Murphy (unpub.)@			6 women	45 ± 4	ext	RTA (human)
			"	39 ± 3	ext	RTA (dog)
			"	33 ± 3	ext	RTA (horse)
			"	14 ± 2	ext + chrom	RTA (dog)

* Lipidex; DIDA = double isotope derivative assay

@ Similar relative differences were obtained for urines obtained from 6 men, 6 pregnant women and 6 children.
Aliquots of urine were extracted with 5 volumes methylene chloride twice. The organic phase was evaporated to dryness and redissolved in 1.0 ml protein-tracer solution. The assay was completed using Florisil as adsorbent (20).

accepted these as true cortisol values. When RIA was first used for urinary cortisol by Ruder et al (7), they obtained a mean of 80 µg/day without chromatography, and about 40 µg/day with chromatography, the latter in apparent agreement with earlier reports (4,5,6). Stahl et al (10) also found good agreement between an RIA (mean 43 µg/day, range 21-91 µg/day) and an RTA

using horse serum (mean 36 μg/day, range 17-79 μg/day). These findings led to the general acceptance of a mean normal value of about 40 μg/day.

However, when we later compared values for a group of urines employing several CBG's we obtained slightly lower values with dog CBG than human with CBG and still lower values with horse CBG. Assay following chromatography revealed that about one-half of the material measured using dog or human CBG was cortisone. Small amounts of progesterone and corticosterone were also included. Two small, slightly more polar peaks appeared consistently which were intermediate in polarity between cortisol and 6 β-hydroxycortisol and may have represented 20 α and 20 β-dihydrocortisol which cross-react with most CBG's and have not been tested for cross-reactivity in RIA. 6 β-dihydroxycortisol, although present in large amounts in urine (Table 2), cross-reacts poorly with all CBG's and again has not been tested for cross-reactivity in RIA. It is poorly soluble in methylene chloride (the solvent most often used to extract urinary cortisol) but the amounts in urine are so large that its effect could be appreciable.

Our values for cortisol RTA after Sephadex LH-20 chromatography using a 42 cm column were very close to those obtained by Chattoraj et al (9) for pregnant and non-pregnant

TABLE 2

SOME UNCONJUGATED STEROIDS IN URINE
Mean ± SEM µg/day

Authors	Ref.	Year	Steroid	Men	Cycling Women	Pregnant Women
Katz et al	(14)	1962	6βOH-cortisol	298	399	845
Minick	(15)	1965	Cortisone	66 ± 6		
Chattoraj et al	(9)	1976	Progesterone			16 ± 3
Murphy	Unpub.*		Progesterone	<5	<5	15 ± 3
"	"		17αOH-progesterone	<5	<5	<5
"	"		Cortisone	74 ± 16	46 ± 6	76 ± 10
"	"		Corticosterone	<5	6 ± 2	8 ± 4
"	"		11-desoxycortisol	<5	<5	<5
"	"		21-desoxycortisol	<5	<5	<5

* Methylene chloride extracts of urine to which tracer steroids had been added were chromatographed on Sephadex LH-20 columns (42 x 0.8 cm). The fractions were assayed using dog serum as the source of CBG and Florisil as adsorbent, using the appropriate steroid as standard (16).

women using both RIA and RTA after thin layer chromatography, and appear to be at least closer to the true values. How then are we to account for the apparent consistency between DIDA, RTA without chromatography, and RIA with chromatography by Ruder et al (7) and by Apter et al (8). The former workers used Sephadex LH-20 chromatography prior to RIA but their 12 cm column may have yielded incomplete separation of cortisol. Chattoraj et al (9), using similar short columns, showed

inadequate separation when compared to TLC. Apter et al (8) used Lipidex and their data may also be explained by incomplete resolution. Even after chromatography, the values of Apter et al were higher than any values by RTA without chromatography. This difference might be due to 20 β-dihydrocortisone which co-elutes with cortisol and is another steroid whose cross-reactivity in RIA is not defined although it is known to be excreted in urine (13); it cross-reacts very little in RTA's (Murphy, unpublished data). The difference might also be due to interference by more polar compounds which would have been extracted into the diethyl ether:ether acetate 1:1 mixture which was used.

From a clinical point of view the absolute specificity of the assay is less important than that it should provide a true reflection of the glucocorticoid production of the patient, i.e. values exceeding the normal range should reliably indicate increased function while those below should indicate decreased function. For this the interfering compounds must change in concert with cortisol, leading to differing but clinically valid normal ranges. Thirteen years personal experience of the human RTA has shown this method to be clinically reliable and although the normal ranges for the dog RTA and horse RTA are slightly lower, the correlation among the 3 methods is excellent. On the other hand, there are few data attesting to

the clinical reliability of most of the cortisol antibodies currently being used for RIA; our own studies with 4 different antibodies available commercially, comparing values in 20 different urines from randomly selected patients, suggest that the results vary appreciably from one antibody to another (Murphy, in preparation). Antibodies to haptens conjugated at C3 gave lower results than with antibodies to haptens conjugated at C21, and the former were more closely correlated to values obtained by RTA. Each individual cortisol antibody must be carefully evaluated before it can be used for urinary determinations. Commercial suppliers should provide detailed cross-reactivity and clinical correlation data for their particular antibody. At present RTA's appear to be more reliable because the specificity of CBG is constant for each species.

The danger of using an unvalidated RIA for urinary determinations became apparent recently in one of our local hospitals where the laboratory supervisor replaced a non-chromatographic RIA kit with another which recommended chromatography for urine samples. No check of the normal range using the new method was made, and no data for urine were provided by the supplier. The supervisor assumed that the normal range was the one they had always used. The discrepancy was picked up only after 6 months when a clinician queried a

series of 'normal' results in a group of severely stressed patients expected to have high values. One would have expected that a control urine should have revealed the difference immediately, but the only control used was serum, for which the usual values were obtained. Since cortisol is stable in frozen urine, it is easy and convenient to freeze small aliquots of several urines (e.g. with high, low, and intermediate values) for use as controls, and to include at least one of these in each assay.

It does appear that the mean non-pregnant normal adult cortisol excretion is 10-20 μg/day with a range up to about 50 μg/day rather than the generally accepted mean of 40 μg/day with a range up to 120 μg/day. Accepting the same range for assays using binding proteins with different specificities may be misleading. None of the available methods has yet been proven to be specific but clinically useful data can be obtained from urine assays provided the interfering substances are minor components and always vary in the same way as cortisol itself. RIA's of poorly defined specificity are unacceptable for use in urine assays.

Serum Cortisol

Although there are differences in serum cortisol values obtained with various RIA's, and RTA's and with use of 3H, 125I

or 75Se as isotopic label, assays on serum are more consistent and better validated than those on urine. There is less clinical reliance on absolute values since the normal diurnal variation is very wide and short term fluctuations may also occur. Serum assays are most useful in evaluating responses to adrenal stimulation or suppression and for this purpose all the RIA's and RTA's are reasonably reliable. Fluorometric assays give generally higher results, particularly in the low range owing to non-specific fluorescence (12). Sera from patients with congenital adrenal hyperplasia and from neonates are most likely to generate inaccurate results.

Congenital Adrenal Hyperplasia

Most cortisol methods, RIA, RTA or fluorescence, have been validated only for normal adult serum containing a fairly well defined mixture of steroids. Since most clinical investigations of adrenal function are carried out on patients with qualitatively similar steroid patterns, these methods suffice. Without prior chromatography, none of these methods is adequate to measure cortisol in patients with congenital adrenal hyperplasia where a different steroid milieu is present. There are large amounts of 21-deoxycortisol, 17 α-hydroxyprogesterone and/or 11-deoxycortisol, which compete in all RTA's and most RIA's; while data for fluorescent methods

are lacking. A rapid screening test for these less polar steroids is to extract them into petroleum ether, with subsequent assay by RTA (20).

Fetal and Neonatal Serum

In recent years there has been considerable interest in studying fetal and neonatal blood and here again the conventional assays are all unsatisfactory. It has been known for 2 decades that fetal serum contains large amounts of steroids and steroid conjugates not found to any extent in the adult. A value of about 80 ng/ml for cortisol in umbilical cord serum at normal delivery was established in 1962 by Bro-Rasmussen et al (18) using DIDA and has been verified by various chromatographic techniques since (see ref. 11 for review). In 1963, we showed that if the RTA using human CBG were to be used on samples from pregnant women, it was necessary to remove progesterone by washing the serum with petroleum ether (1); this was later shown to be unnecessary when using certain batches of Fuller's earth as adsorbent to separate the bound and unbound fractions (20) or when using horse serum as the source of binding-protein (16). Klein and Giroud (34) pointed out in 1973 that the RTA using human CBG gave high values in neonates so that chromatography was necessary. In 1975 I showed that if horse serum is used a wash

and extraction can be substituted for chromatography (16) (see Table 3).

Cross-reactions using RIA for cord serum are even more pronounced than for RTA because all of the available antisera raised against cortisol conjugated at the 21-position cross-react strongly with cortisol sulfate, and usually also with corticosterone sulfate and desoxycorticosterone sulfate, all of which are present in appreciable amounts (35). Many authors have not attempted to validate their methods by chromatography, relying entirely on cross-reactivity data which do not include the competing compounds (e.g. 29,30). Thus while the mean value of mixed umbilical cord cortisol after normal term vaginal delivery is well established to be about 80 ng/ml, mean values as high as 437 ng/ml have been reported (28) within the past few years. High values have also been obtained using fluorometric methods, which have not been adequately validated for cord serum. As long as the fetal and neonatal steroid pattern remains incompletely characterized, it is essential to investigate each method by careful chromatographic studies.

Even with prior Lipidex chromatography it appears that the RIA used by Apter et al (8) for urine and by Kauppila (31) for cord serum (see Tables 1 and 3) gave high results. As with the

TABLE 3

MEAN SERUM CORTISOL LEVELS IN UMBILICAL SERUM

AT NORMAL TERM VAGINAL DELIVERY

Mean ± SEM ng/ml

Authors	Year	Ref.		Cortisol	Purification	Assay
Ulstrom et al	1961	(17)		86	Multiple chr.	DIDA
Bro-Rasmussen et al	1962	(18)		92	" + DF	DIDA
Hillman & Giroud	1965	(19)		78	" + DF	DIDA
Murphy & Diez d'Aux	1972	(21)		70 ± 4	ext + chr	RTA (dog)
Turner et al	1973	(22)	A V	73 ± 12 49 ± 10	ext + chr	RTA (human)
Murphy & Leong	1976	(23)	A V	79 ± 4 62 ± 4	wash + ext	RTA (horse)
Pokoly	1973	(24)		89 ± 5	ext + chr	RTA (human)
Cawson et al	1974	(25)	A V	158 ± 25 166 ± 14	ext + chr	RTA (human)
Sybulski et al	1976	(26)		74 ± 3	ext + chr	RTA (human)
Weekes et al	1976	(27)		76 ± 12	ext + chr	
Goldkrand et al	1976	(28)		437 ± 14	ext only	RTA (human)
Knapstein et al	1975	(29)	A V	285 ± 26 306 ± 30	none	RIA
Ohrlander et al	1976	(30)	A V	147 ± 8 151 ± 11	ext only	RIA
Kauppila et al	1976	(31)		180 ± 9	ext + chr*	RIA
Toaff et al	1974	(32)	A V	162 ± 10 181 ± 11	ext only	Fluorometry
Roopnarinesingh et al	1977	(33)		214 ± 11	ext only	Fluorimetric

DIDA = double isotope dilution derivative assay; A = arterial; V = venous; *Lipidex chromatography; ext = extraction; chr = chromatography.

similar LH-20 chromatography, use of a short column with limited resolution probably led to interference by other steroids. Ohrlander et al (30) using an RIA validated by Sephadex LH-20 chromatography (column length not given) obtained values about twice ours. Since even a 50% increase in the cortisol value due to steroids arising from the placenta would obscure the positive arterial-venous differences observed by Turner et al (22) and by us (23) (see Table 3), the data obtained by the less specific methods may be misleading and confusing.

All 3 of the RIA's quoted in Table 3 used antibodies conjugated at the 21 position. More recently Gomez-Sanchez et al (36) described an antibody to cortisol linked at the 3-position. As used by Hauth et al (37) without prior chromatography, this antibody gave values for cord serum of term and premature infants of 95 $\pm$ 7 ng/ml compared to our values of 47 $\pm$ 9 ng/ml for a comparable group of infants (this data is not included in Table 3 because the authors did not quote values for normal term vaginal deliveries). For infants who developed RDS, their value was 70 $\pm$ 6 ng/ml compared to our value of only 24 $\pm$ 4 ng/ml. Since the method was validated by chromatography only for adult serum from non-pregnant subjects it seems likely that other steroids cross-react in cord serum in this type of RIA as well.

With the exceptions of Kauppila et al (31) and Cawson et al (25), all the DIDA and RTA methods which included chromatography (8 different groups) had closely similar values which were substantially lower than those using RIA or fluorometry.

Amniotic Fluid Cortisol

With the increasing use of amniocentesis in early pregnancy for intra-uterine diagnosis of genetic disorders and in late pregnancy for assessment of lung maturation there have been at least 12 reports on amniotic fluid cortisol. Many workers have again assumed without validation that what will serve for adult serum will do equally well for amniotic fluid.

As shown in Table 4, Baird and Bush (38) using DIDA demonstrated in 1960 that cortisol levels in amniotic fluid at term were very low. In 1975 3 papers appeared with radically different results - our own using RTA which agreed with Baird and Bush and 2 RIA's with much higher values being obtained. In one of these, Fencl and Tulchinsky (44) found that extraction reduced their values and therefore called their unextracted values 'total' cortisol, assuming that the unextracted residue was conjugated cortisol. We later found that while much of the material was indeed cortisol sulfate, corticosterone sulfate, which cross-reacted with their antibody, also contributed (49).

TABLE 4

AMNIOTIC FLUID CORTISOL LEVELS AT TERM
Mean ± SEM ng/ml

Authors	Ref.	Year	Cortisol	Purification	Assay
Baird & Bush	(38)	1961	26 ± 3	Ext chrom DF	NaOH Fluor*
Murphy et al	(16,39)	1975	22 ± 3	Ext + chrom	RTA (horse)
Murphy et al	"	1975	22 ± 3	Ext	RTA (horse)
Murphy et al	(40)	1977	27 ± 4	None	RTA (horse)
Tan et al	(41)	1976	23 ± 3	Ext + chrom	RTA (human)
Nwosu et al	(42)	1977	27 ± -	Ext	RTA (dog)
Sivakumaran et al	(43)	1975	202 ± 13	None	RIA
Fencl & Tulchinsky	(44)	1975	139 ± 12 28 ± 4	None ('total') Ext	RIA RIA
Peltonen et al	(45)	1977	20 ± 2	Ext + chrom	RIA
McCann et al	(46)	1977	70 est.	None ('total')	RIA
Gennser et al	(47)	1976	33 ± 3	Not stated	RIA
Brazy et al	(48)	1978	43	Wash + ext	Fluorometry

Fluorescence, estimated visually

We also found that RTA using horse serum without extraction or chromatography of amniotic fluid gave only slightly increased values over those obtained with either extraction alone or with extraction followed by chromatography (40). Other authors have

confirmed these low values (38,41,42,45). From Table 4 it is apparent that results using RIA are more variable than those using RTA and that with RIA, prior extraction is a minimum requirement for measurement of unconjugated cortisol. Our data suggest that the cortisol sulfate in amniotic fluid probably provides a better index of fetal adrenal secretion than does cortisol itself (49) since cortisol enters amniotic fluid via the membranes as well as the fetal urine (50). It is, therefore, inappropriate to assay the two compounds as 'total cortisol'.

SUMMARY

Some of the problems in clinical usage of cortisol assays in urine, blood and amniotic fluid have been discussed. In the early '60's fluorometric methods give higher values for most cortisol assays, and these methods are still in use. The use of antibodies to cortisol in a variety of hapten linkages in RIA's has not provided the specificity required for some biological fluids and for most clinical purposes has been less satisfactory than the use of transcortin in RTA's. Current problems may be solved by changes in the hapten linkage used. Until then careful validation of each method is essential on the specific biological fluid and using samples from disease states of interest.

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