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Note

Three-hour assay for urinary free cortisol: historical comparison of three methods each using a different chromatographic procedure

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The measurement of urinary free cortisol is used to assess the 24-h steroidal function of the adrenal gland. Assay methods have quantitated the cortisol by using double isotope derivative [1], fluorimetric [2], protein binding [3], radioimmunoassay (RIA) techniques [4] and, more recently, employing high-performance liquid chromatography (HPLC) [5–8]. Over the past several years it has been our experience, however, that the most important and in fact essential part of the assay is the purification of the cortisol prior to its actual quantification. The length of time of the chromatographic procedure varies with the method used and, therefore, can effect to a considerable extent the turn-around time of the assay, a very important factor in performing clinical determinations.

We have recently incorporated a rapid chromatographic purification with a completely automated RIA system for the assay of urinary free cortisol. The results from this method show excellent correlation with results from two other methods previously used in our laboratory and produce a several-fold decrease in turn-around time.

EXPERIMENTAL

Materials

The HPLC system used consisted of a Vista 401 chromatography data system, Vista 5040 terniary liquid chromatograph (Varian Instruments, Sunnyvale, CA, U.S.A.), WISP automatic sample injector, Sep-Pak cartridge rak, 10 μm particle

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size, μBondapak C_{18} column, 25 cm × 0.46 cm I.D. (Waters Assoc., Milford, MA, U.S.A.) and a Foxy automatic fraction collector (Isco, Lincoln, NE, U.S.A.). The sample buffer 1 is supplied by Becton Dickinson Immunodiagnostics (Salt Lake City, UT, U.S.A) for use in their automated RIA system instrument ARIA II, as was the cortisol antibody. Other reagents used were [^{3}H]cortisol, 40–50 Ci/mmol, >98% radiochemically pure by HPLC, obtained from New England Nuclear (Boston, MA, U.S.A.), methanol, methylene chloride, spectroquality reagent grade, from Fisher (Medford MA, U.S.A.) and acetonitrile, water, HPLC grade, from Baker (Phillipsburg, NJ, U.S.A.)

Extraction

The extraction of urine was the same for all three methods. Tritiated cortisol (3000 dpm, method 1, 14 000 dpm, methods 2 and 3) was added to 4 ml of a 24-h urine collection. The urine was extracted by shaking with 10 ml of methylene chloride, the extract washed with 1 ml of water twice and then evaporated to dryness in a glass vial (in vacuo, 55°C) for chromatographic purification.

Method 1: paper chromatography and manual RIA

The paper chromatographic purification, RIA, liquid scintillation counting procedures and computation of cortisol in μg/dl were as previously reported for plasma cortisol by Underwood and Williams [9]. The excretion of free cortisol in μg per 24 h was calculated using the formula:

$$\mu\text{g per 24 h} = \frac{\mu\text{g/dl} \times V_{\text{tot}}\ (\text{ml})}{400}$$

where V_{tot} is the total volume of urine collected in 24 h.

Method 2: HPLC and automated RIA

HPLC. A 0.20-ml volume of a mixture of 98% acetonitrile–water was added to the dried extract and 0.18 ml of the solution were analysed by HPLC. The column was eluted with 98% acetonitrile–water, at a flow-rate of 1 ml/min, and the eluate collected in 1-min fractions in an automatic fraction collector. Cortisol appeared in tubes 5 and 6. The contents from these tubes were combined and evaporated to dryness in a glass vial (in vacuo, 55°C).

Automated RIA. A 0.5-ml volume of buffer 1 was added to the dried eluate in the glass vial.

(i) Recovery determination. To determine the recovery of the entire procedure 0.05 ml of the solution were transferred to a plastic vial (recovery vial), 4 ml scintillation fluid added and the tritium was assayed in a liquid scintillation counter.

(ii) RIA. A 0.2-ml volume of the solution was transferred to a 1-ml plastic cup for RIA in an ARIA II instrument that uses an isokinetic continuous flow system with a reusable antibody chamber. An aliquot of the sample is aspirated, mixed with [^{125}I]cortisol and introduced into the antibody chamber. The antibody chamber contains cortisol-specific antibody covalently bound to a solid support medium. The binding is virtually instantaneous and the free [^{125}I]cortisol flows through the chamber and is counted. Buffer, which releases the bound

[^{125}I]cortisol, is pumped through the chamber and the released bound [^{125}I]cortisol is also counted. Both free and bound counts are correlated with a known amount of total counts and the instrument computes the level of cortisol in the solution in the plastic cup in μg/dl. The standard curve has a range from 0 to 64 μg/dl cortisol, and the lowest level of cortisol which can be assayed by this procedure is 2.5 μg/l of urine.

The cortisol in μg per total volume urine is calculated using the formula:

$$\mu g \text{ per } 24 \text{ h} = \frac{\mu g/dl \times V_{tot} \text{ (ml)} \times I}{2000 \times U \times R}$$

where I = cpm [3H]cortisol added to the urine initially; U = ml urine extracted; R = cpm recovery vial; V_{tot} = total volume of urine collected in 24 h.

Method 3: Sep-Pak chromatography and automated RIA

Sep-Pak chromatography. Sep-Pak was activated by pushing through it from a syringe 5 ml methanol, then 10 ml water. Water (1 ml) was added to the dried extract in the glass vial and the solution pushed through the Sep-Pak from a syringe. The Sep-Pak was then eluted successively with 5 ml of water, 3 ml of 20% methanol–water and 2 ml of 100% methanol. The last eluate contains the cortisol and was evaporated to dryness in a glass vial (in vacuo, 55°C).

Automated RIA for cortisol. This was carried out the same as in method 2.

RESULTS

Comparison of recoveries, values and assay time for the three methods

The mean (±coefficient of variation, C.V.) recovery of [^{3}H]cortisol internal recovery indicator added to the urine, after extraction and chromatography was: method 1, 55±10.2% (n=20); method 2, 72.1±4.6% (n=20); method 3, 74.5±6.7% (n=50). A comparison of values obtained using the three methods is shown in Table I. A least-squares regression analysis of results from methods 1 and 2 and methods 2 and 3 indicated correlation coefficients of 0.98 and 0.97, respectively ($p<0.01$), thus indicating no significant difference between the results at the 99% confidence limits. The normal range was the same for all three methods, i.e. 20–100 μg per 24-h urine.

The length of time for the assay was five days, one day and 3 h for methods 1, 2 and 3, respectively. The timetable for a batch of eight samples processed in method 3 is as follows: extraction of urine with methylene chloride, 40 min; drying extract, 40 min; Sep-Pak chromatography, 25 min; drying the methanol eluate, 30 min; RIA and computation in ARIA II, 45 min, total time, 180 min.

Precision and accuracy

The precision and accuracy of method 1 were similar to that for plasma cortisol reported by Underwood and Williams [9]. The precision and accuracy of methods 2 and 3 are shown in Tables II and III, respectively. To determine accuracy, given amounts of standard cortisol (0, 20, 40 and 80 μg per V_{tot}, respectively) were added to aliquots of urine. The samples were assayed for cortisol and the value for the endogenous level of cortisol (0 μg added) was subtracted from the

TABLE I

URINARY FREE CORTISOL VALUES OBTAINED COMPARING METHODS 1 AND 2 AND METHODS 2 AND 3

Sample No.	Cortisol level ($\mu g/V_{tot}$)		Percentage difference	Sample No.	Cortisol level ($\mu g/V_{tot}$)		Percentage difference
	Method 1	Method 2			Method 2	Method 3	
1	172	190	10	1	24	23	4
2	18	29	61	2	36	47	28
3	8	8	0	3	11	12	9
4	19	22	16	4	65	88	35
5	9	8	11	5	36	38	6
6	24	29	21	6	40	41	2
7	3	3	0	7	11	13	18
8	130	105	19	8	23	19	17
9	73	89	22	9	19	23	21
10	225	228	1	10	29	24	17
11	89	72	19	11	18	18	0
12	62	40	35	12	127	116	9
13	71	99	39	13	80	79	1
14	54	58	7	14	46	66	43
				15	62	73	18
				16	29	18	38
				17	17	19	12
				18	21	25	19
				19	113	118	4
				20	42	34	19
Mean	68.4	70.0	18.6		42.5	44.7	16.0
S.D.			17.0				12.5
C.V. (%)			91.4				79.9

levels found (endogenous plus added) to give the recovery of the micrograms added. The recovery ranged from 91 to 108%. These values are within the precision range of the method, 7–11%, thus indicating good accuracy for the procedure.

TABLE II

PRECISION OF METHODS 2 AND 3

Method	Mean cortisol level ($\mu g/V_{tot}$)	Coefficient of variation (%)
Intra-assay precision (n=6)		
2	62	11
3	24	7
Inter-assay precision (n=10)		
2	58.9	14.2
3	40.2	5.3

TABLE III

ACCURACY OF METHODS 2 AND 3

The recovery of μg standard amounts of cortisol added to urine.

Method	Cortisol added ($\mu g/V_{tot}$)	Cortisol measured ($\mu g/V_{tot}$)	Cortisol recovered ($\mu g/V_{tot}$)	Average recovery (%)
2	0	41	0	–
	23	67	25	108
		65	24	
	46	86	45	96
		84	43	
	80	115	74	91
		113	72	
3	0	24	0	–
	20	46	22	90
		38	14	
	40	61	37	90
		59	35	
	80	109	85	100
		100	76	

DISCUSSION

That urinary cortisol must be chromatographically purified before assay has been firmly established in a detailed investigation of the specificity of urinary free cortisol determinations by Murphy et al. [10]. They showed that, without a chromatographic step, the levels are grossly overestimated. This has also been our experience with urinary cortisol assay over the last twelve years. During this period, the three methods described, each using a different chromatographic procedure, superseded each other in our laboratory, thus we could not directly compare all three methods at once, but only the method and the one which was replacing it, i.e. methods 1 and 2 and later 2 and 3. Also due to the time difference, a different set of control samples had to be used for the two comparisons. The Sep-Pak is finding increasing usage for the extraction and/or initial purification of hormones and other compounds from biological fluids. We found that the cortisol had to be extracted into an organic solvent prior to Sep-Pak chromatography in order to achieve sufficient purification for values to have no significant difference from values obtained using more lengthy paper chromatographic and HLPC procedures. The antibody has a cross-reactivity of 16.1% with cortisone and 3.3% with corticosterone. These steroids are not removed by Sep-Pak chromatography but occur in such trace amounts in urine relative to cortisol that they would not interfere significantly with the assay. In the HPLC purification procedure in method 2, the system was designed to give a rapid 6-min purification step which adequately removed non-specific impurities which interfered with the antibody binding in the RIA, thus giving fictitiously high cortisol values. In attempting to quantify the cortisol on line with a UV detector in the HPLC system we found

considerable variation from urine to urine of interfering UV-absorbing compounds. However, several HPLC methods with adequate accuracy and specificity have been published for the assay of urinary cortisol [5–8].

The extraction procedure was the same for all three methods. In methods 2 and 3 the RIA was automated, thus it was the different type of chromatography used which accounted for the difference in the length of the assay. In addition, the use of a Sep-Pak cartridge rak in method 3 enabled eight Sep-Pak assays to be performed simultaneously. We also found that by washing a Sep-Pak immediately after use, consecutively with 10 ml of water, then methanol and then storing it in methanol, it could be used for six consecutive assays. The use of manual RIA in method 3 instead of automated RIA would still greatly reduce the turn-around time for a urinary free cortisol assay, which could be a desirable advantage for a clinical procedure.

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