The present scheme permits the best possible slope estimates with the fewest dilutions. Excessive numbers of dilutions are impractical for routine clinical use and would not prevent underestimation of slope caused by the presence of weakly cross-reacting substances (identified or unidentified) in the sample.

Initial clinical toxicology results obtained by immunoassay are reported as “presumptive”. This study was carried out to determine whether the use of sample dose–response characteristics could strengthen this presumption. As far as we know, this study is the first of its kind to examine the utility of such an approach. We chose to first investigate the properties of amphetamine screening assays because the target compounds are few and many of the potential cross-reactants are well defined. The technique displayed considerable power to identify specimens containing (meth)amphetamine. In our data set, the PPV of the immunoassay to detect amphetamine abuse without the dilution protocol was 57%. Use of the dilution protocol increased the PPV to 92%. Our dilution approach cannot, however, guarantee a 100% negative predictive value. High concentrations of less reactive substances may reduce the dose–response slope in rare specimens and mask the presence of low amphetamine concentrations.

Widespread use of this sample dose–response approach in other commercial amphetamine immunoassays poses some obstacles. Antibody cross-reactivity is variable; therefore, slope cutoffs will be assay specific. Manufacturers would likely have to define acceptable cutoffs for laboratories without access to drug standards on a lot-specific basis. The utility of this approach in immunoassays for other drugs of abuse (e.g., opiates, benzoylcegonine, cannabinoids, and phencyclidine) will depend on the selectivity of the antibodies, the degree to which similar cross-reacting drugs are present in urine specimens, and the degree to which unchanged parent drug is excreted in the urine. Extensive conversion of parent compound to less reactive metabolites will decrease the ability of sample dose–response characteristics to distinguish highly reactive from less reactive substances in urine.

We have shown here that sample dose–response characteristics can distinguish highly reactive amphetamines from moderately reactive compounds (e.g., MDMA) and weakly reactive compounds (e.g., pseudoephedrine) in urine samples that contain a single species of drug. Clinical urine specimens, however, contain mixtures of these compounds; consequently, slope values reflect the relative concentrations of cross-reacting compounds and their relative affinity for antibody. The value of the approach described here is to increase the PPV of the initial presumptive result in a time frame more rapid than is typically available when GC/MS is used. In a pediatric emergency setting, this technique may quickly indicate that a urine specimen contains illicit amphetamines rather than an over-the-counter cold remedy, information that might prevent patient discharge and return to a suspected abusive environment before the results of GC/MS analysis are completed. It must be emphasized, however, that this dilution protocol is not intended to replace the need for confirmation of immunoassay results by a more definitive method such as GC/MS, particularly in workplace or forensic settings.

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References


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Relationship between Cortisol Increment and Basal Cortisol: Implications for the Low-Dose Short Adrenocorticotropic Hormone Stimulation Test, Suhail A.R. Doi,7 Ibrahim Lasheen,2 Khaldoon Al-Humood,2 and Kamal A.S. Al-Shoumer3 (Divisions of 1 Endocrinology and 2 Medicine, Mubarak Al Kabeer Teaching Hospital, Jabiya, Kuwait; *address correspondence to this author at: Division of Endocrinology, Mubarak Al Kabeer Teaching Hospital, PO Box 64849 Shuwaikh, 70459 Kuwait; fax 44-709-2377990, e-mail sardoi@gmx.net)

Background: We analyzed the low-dose (1 µg) rapid adrenocorticotropic hormone test (LDST) in 17 patients with a normal hypothalamic-pituitary-adrenal axis to determine reference intervals for the LDST on the basis of poststimulation cortisol increments.
Methods: We analyzed test results for 17 patients (14 females and 3 males; age range, 18–46 years) who had received a 2-mL aliquot of low-dose (1 µg) adrenocorticotropic hormone prepared from one 250-µg vial of Synacthen diluted in 500 mL of sterile normal saline solution. Sampling took place at 0, 20, 30, and 60 min post stimulation. The cortisol increment was plotted against basal cortisol.

Results: We observed a marked interdependence of the basal cortisol concentration with the increase in cortisol concentration. The relationship was inverse and linear with the best fit observed at 30 min post stimulation. The lower 95% prediction limit for basal cortisol at the zero increment was 400 nmol/L with a mean concentration of 600 nmol/L.

Conclusions: We propose that a peak cortisol concentration <400 nmol/L is a sufficient single criterion for abnormal adrenal function as assessed by the LDST. Concentrations of 400–600 nmol/L are in the gray area, and those >600 nmol/L confirm normal adrenal function. Repeat analyses with larger sample sizes are warranted to confirm these observations.

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The standard short high-dose (250 µg) adrenocorticotropic hormone (ACTH) stimulation test (HDST) with synthetic ACTH is the most widely used test for the detection of primary or prolonged secondary adrenocortical insufficiency (1). Whether this test accurately predicts early secondary adrenal insufficiency (2, 3) is an area of ongoing controversy because occasionally patients have a normal response to the HDST but a subnormal response to insulin-induced hypoglycemia (IIH), a test considered the gold standard test for evaluation of the hypothalamic-pituitary-adrenal (HPA) axis. The pharmacologic dose of 250 µg is useful for assessing the maximum secretory capacity of the adrenal cortex (1), but this dose may be excessive for the assessment of subclinical central hypocortisolism. The low-dose short ACTH stimulation test (LDST) has been shown to correlate well with IIH in such cases (4–7). Several centers are already using the LDST, and the higher sensitivity of the LDST in the diagnosis of mild forms of secondary adrenal insufficiency is already known (4, 8, 9). The LDST is also a sensitive test for mild forms of primary adrenal insufficiency (10) but is not more sensitive than the HDST, as demonstrated by sequential LDST-HDST studies (11).

Previous studies have evaluated the operating characteristics of the LDST in adult patients with normal and abnormal adrenal function. On the basis of the results of these studies, a serum cortisol concentration of 500 nmol/L (18 µg/dL) or higher (12, 13), before or after low-dose ACTH injection, is suggested to be indicative of normal adrenal function. Although retrospective analysis of the HDST in a large adult population showed a marked interdependence of the basal cortisol concentration, peak cortisol concentration, and increases in serum cortisol concentration (14), criteria that require a minimum increment in serum cortisol (13) are considered invalid because individuals who have a lower basal serum cortisol concentration because of recent ACTH deficiency may be maximally stimulated by the HDST and thus able to further increase cortisol secretion. The increment with a more physiologic (1 µg) dose may be expected to correlate better with the degree of adrenal stimulation. Persons with high-normal basal serum cortisol concentration may therefore have little or no increase after ACTH stimulation, whereas those with low-normal basal serum cortisol may have a maximal increase. With the HDST, however, this increase may still occur if the HPA axis is compromised. We therefore decided to investigate the relationship between the peak and basal cortisol values after the LDST.

We studied 17 patients (14 females and 3 males; age range, 18–46 years) with suspected primary adrenal dysfunction that was subsequently ruled out via a normal cortisol response on the HDST. The patients were followed in the endocrinology clinic and underwent ACTH stimulation testing to exclude 21-hydroxylase deficiency and primary adrenal dysfunction suspected in the presence of other autoimmune disorders (such as hypothyroidism or diabetes mellitus). No patient was suspected of having ACTH deficiency, and none of the female patients was pregnant or taking oral contraceptives. Each patient was tested with the HDST, and if a normal result was obtained [peak >550 nmol/L (20 µg/dL)], then (within 1–2 weeks) with the LDST. We prepared low-dose (1 µg) ACTH in 2-mL aliquots with one 250-µg vial of Synacthen diluted in 500 mL of sterile normal saline solution. We obtained patient serum samples at 0, 20, 30, and 60 min after ACTH administration for the LDST. Each serum sample was separated and preserved in deep freezer at −20 °C in the laboratory until analysis. Plasma cortisol was determined by the DSL-2100 cortisol-coated tube RIA (DSL). The study was done after we obtained informed consent from all participants.

The basal cortisol concentrations varied widely (Fig. 1A), from 149 to 685 nmol/L (5.4–24.8 µg/dL); the mean (SD) concentration was 422.4 (138.2) nmol/L. The stimulated cortisol correlated inversely and linearly with basal cortisol, and the best correlation between basal and stimulated cortisol was at 30 min, with a linear relationship between the proportional increase in cortisol (30-min cortisol/basal cortisol) and the basal cortisol (Fig. 1B). The 95% prediction limits obtained by linear regression analysis are shown in Fig. 1B, and the cutoff for the lower 95% prediction limit at a stimulated/basal ratio of 1 was ~400 nmol/L (14.5 µg/dL).

The false-negative rate for the LDST has been reported to be close to zero at cutoffs above 500–550 nmol/L (18–20 µg/dL) (4, 5, 7, 15–17). The LDST has also been advocated as a more sensitive test for ACTH deficiency than the HDST (18), but use of the same cutoff points for the LDST and HDST would serve to increase the apparent sensitivity of the LDST because peak cortisol responses are less after 1 µg than 250 µg of ACTH (19). At this cutoff
point, however, false-positive rates are high, leading to low specificity (19).

We demonstrate here that the cortisol increment after LDST is inversely related to the basal cortisol, strongly suggesting that the peak cortisol depends on the state of adrenal stimulation at the time of the test. False positivity occurs because in many patients peak cortisol concentrations <500–550 nmol/L (18–20 μg/dL) represent normal variability, which has a wide range in humans, and not adrenal insufficiency. The incremental response to 1 μg of ACTH, however, is directly and inversely dependent on basal cortisol concentrations, decreasing to zero at a mean basal cortisol concentration of 600 nmol/L (21.7 μg/dL; Fig. 1B).

When devising a screening test, one aims for maximum sensitivity in the face of a reasonable specificity—in other words, excluding false negatives without including too many false positives. However, as is clear from Fig. 1B, with the LDST, the lowest basal cortisol concentration at which no increment may occur after stimulation is 400 nmol/L (14.5 μg/dL). This characteristic indicates that 100% sensitivity will be associated with markedly poor specificity, limiting the use of the LDST in this way. We therefore suggest that the lower 95% confidence interval for the basal cortisol at which the increment is zero be taken as the cutoff value associated with zero false positives. Any value <400 nmol/L (14.5 μg/dL) after LDST will therefore be abnormal. Any value above this and up to 600 nmol/L (21.7 μg/dL), our mean value at zero increment, needs a confirmatory test such as IIH or overnight metyrapone testing because the false-negative rate could be as high as 50% (19). Above 600 nmol/L (21.7 μg/dL), the test result is highly specific and need not be confirmed. Our gray zone is wide because of our small sample size; thus we recommend that this analysis be repeated with a larger sample size to obtain a more definitive gray zone with a lower upper limit.

References
15. Ambrosi B, Barletta L, Re T, Passini E, Faglia G. The one microgram


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Total Polyphenol Intake Estimated by a Modified Folin–Ciocalteu Assay of Urine, Elena Roura, Cristina Andrés-Lacueva, Ramon Estruch, and Rosa M. Lamuela-Raventós

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Background: Plant polyphenols have been studied largely because of the possibility that they might underlie the protective effects afforded by fruit and vegetable intake against cancer and other chronic diseases. Measurement of polyphenol content excreted in urine as an indicator of polyphenol consumption may offer a routine screening method that could be used for these pathologies.

Methods: Thirty-six healthy volunteers each received 2 interventions, one with a polyphenol-rich food (cocoa beverage) and one with a polyphenol-free food (milk) as a control, in a randomized cross-over design with 1-week intervals. The total polyphenol content excreted in urine during the 6 h after consumption of the test meals was measured by a modified Folin-Ciocalteu assay after sample cleanup by solid-phase extraction.

Results: The mean (SD) concentrations of polyphenols excreted in the urine 6 h after consumption of the test meals differed significantly: 140.95 (49.27) mg catechin/g of creatinine after the polyphenol-rich meal vs 90.43 (46.07) mg catechin/g of creatinine after the control meal (P < 0.05).

Conclusions: This method allows analysis of a large number of samples per day, which is ideal for use in epidemiologic studies and may enable estimation of polyphenol consumption and determination of their possible role in preventing of certain pathologies, such as cancer, cardiovascular and degenerative diseases.

The idea that the health benefits associated with consumption of fruits, vegetables, tea, cocoa, and red wine are probably linked to polyphenol content has been supported by several recent studies (1–4). The Folin–Ciocalteu (F-C) assay has for many years been used to measure of total phenolics in natural products (5, 6). Since introduction of the improvements recommended by Singleton and Rossi (7), reduction of phenols has also become more specific (8, 9). Nonetheless, there are limited reports describing use of this procedure for biological samples (10–13).

A wide range of water-soluble compounds are typically present in urine, although other substances, such as proteins, glucose, erythrocytes, and ketones bodies, can also be found when the body’s processes are not operating efficiently (14). The F-C assay is affected by several interfering substances, such as sugars, aromatic amines, sulfur dioxide, ascorbic acid, organic acids, and Fe(II), as well as nonphenolic organic substances that react with the F-C reagent (15, 16). We report here the use of a solid-phase extraction (SPE) procedure to remove such water-soluble compounds from urine samples. Combining this SPE with the Singleton and Rossi F-C assay (7), with certain modifications, provides an effective technique for quantifying the total polyphenols excreted in urine; these results can then be related to polyphenol intake. To study this we selected cocoa powder, a food rich in polyphenols. Cocoa and its derived products contain a diverse mixture of flavonoids, such as anthocyanins, flavonols (quercetin and its glycosides), and flavan-3-ols (epicatechin, catechin, and related proanthocyanidin oligomers) (17).

Reagents were obtained from the following sources: methanol (HPLC grade) from Scharlauf; F-C reagent from Panreac, formic acid, caffeic acid, (+)-catechin, gallic acid, and quercetin from Sigma; picric acid solution and creatinine from Fluka, Biochemika; and tyrosol and naringin from Extrasyntese. All chemicals used were of analytical or chromatographic grade. The water was purified in a MilliQ water purification system. The composition of the cocoa power was determined as described by Andrés-Lacueva et al. (18); the flavonoid concentrations for the cocoa powder portion used in this study were 56.4 mg of (−)-epicatechin, 51 mg of procyanidin B2, 16.8 mg of catechin, and 4 mg of flavonols, including isoquercitrin, quercetin, quercetin-glycoside, and quercetin-arabino-side. Synthetic urine was prepared as described by Miró-Casas et al. (19) to avoid any possible interference generated by the matrices with F-C reagent. A series of (+)-catechin calibrators with concentrations of 1, 2, 4, 6, 8, 10, and 12 mg/L was prepared in this synthetic urine. Calibrator preparation and sample processing were performed in a darkened room with a red safety light to avoid oxidation of analytes.

This randomized, crossover trial included 36 healthy adult volunteers (16 women and 20 men; age range, 18–49 years); all were nonsmokers with no history of heart disease or homeostatic disorders. The study was carried out in accordance with the Helsinki Declaration of 1975, as revised in 1996, and the protocol was approved by the