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Short Communication

Fluorimetric detection of serum corticosterone using high-performance liquid chromatography

Steven R. Mason, Leigh C. Ward and Paul E. B. Reilly

Department of Biochemistry, Molecular Biosciences, University of Queensland, Brisbane, Queensland 4072 (Australia)

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ABSTRACT

A simple, sensitive, specific and reproducible method for the determination of corticosterone concentrations in rat serum using high-performance liquid chromatography (HPLC) with fluorimetric detection is described. Corticosterone is detectable down to 0.1 ng injected onto the HPLC column. Cortisol is used as an internal standard. Ethyl acetate was used for both initial serum corticosteroid extraction and the subsequent fluorophore extraction after sulfuric acid hydrolysis; thus sulfuric acid does not enter the HPLC system. The resultant fluorophores for both corticosterone and cortisol are stable for at least two weeks at ambient temperature not requiring storage at −20°C. The procedure is highly suitable for use with HPLC systems utilising automatic sample injectors. The method is specific for corticosterone; dexamethasone, cortisone and gonadal steroids are not detectable and do not interfere in this assay.

INTRODUCTION

High-performance liquid chromatography (HPLC) is a widely used alternative to radioimmunoassay [1–4], spectrofluorimetry [5–7] and gas chromatographic–mass spectrometric techniques [8] for the detection and quantification of corticosteroids. Detection may be by fluorescence [9,10], UV absorbance [11–14] or chemiluminescence [15]. Although HPLC methods using UV detection offer simplicity, HPLC with fluorimetric detection provides the greatest sensitivity and specificity, especially with small sample volumes and trace levels of corticosteroids. Spectrofluorometric detection alone is insufficient, as separation of possible interfering compounds is not possible and variations in extraction efficiencies cannot be corrected.

The basic HPLC–fluorimetric detection method for corticosteroids, *e.g.* Gotelli *et al.* [9], involves extraction of aqueous samples with organic solvent, acid hydrolysis, with or without evaporating the extractant, isolating the acid sample and diluting with HPLC mobile phase. This mixture is then immediately stored at −20°C before being injected onto the HPLC column. Many recent adaptations of the acid-induced fluorescence method add procedural complications and equipment which may be unavailable to laboratories. These include post-column hydrolysis [10] and injector-mounted extraction columns [16].

The present method combines simplicity, high

Correspondence to: Steven R. Mason, Department of Biochemistry, Molecular Biosciences, University of Queensland, Brisbane, Queensland 4072, Australia.

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sensitivity, reproducibility and specificity. By the addition of an ethyl acetate extraction step after acid hydrolysis of samples, acid is removed from the HPLC system and ambient temperature-stable fluorophores are extracted. Immediate storage of samples at −20°C is not required. The method is therefore highly suitable for use with HPLC systems incorporating automatic sample injectors. Methylene chloride and acetonitrile (common to most published methods) are not used in the present method as both pose potential health hazards. Furthermore, methylene chloride is unsuitable for fluorophore extraction after acid hydrolysis. Ethyl acetate was the solvent of choice for both corticosteroid and fluorophore extraction after sulfuric acid hydrolysis.

EXPERIMENTAL

Chemicals

Dexamethasone, corticosterone, cortisol and cortisone were purchased from Sigma (St. Louis, MO, USA). HPLC-grade ethyl acetate and methanol were obtained from Mallinckrodt (Clayton, Australia). Penthrane (methoxyflurane U.S.P.) was purchased from Abbott Labs. (Sydney, Australia). All other chemicals and solvents were of reagent grade, and water was purified using an Elga Spectrum System (conductance of 0.07 μS/cm).

Animals and treatment

Random outbred male and female Wistar rats were obtained from the Central Animal Breeding Unit (University of Queensland, Brisbane, Australia). Animals were maintained on a commercial rodent diet with water *ad libitum* and at 24 + 2°C with a 12-h light–dark cycle. All blood samples, taken at time of sacrifice, were taken 1 h after the start of the light period, collected into centrifuge tubes, centrifuged to obtain serum and stored at −45°C. Adrenalectomy was carried out by the technique described by Waynforth [17].

Sample and standard preparation

Cortisol and corticosterone standard solutions were prepared in methanol at 0.5 mg/ml and diluted in water for working standards. Calibration standards were prepared by adding 50 μl of water containing corticosterone (2.5–500 ng) to 50 μl of adrenalectomized (ADX) serum or 5% bovine serum albumin (BSA) in water. To serum samples (50 μl) and standards (50 μl) in 1.5-ml microcentrifuge tubes, 100 μl of water containing internal standard (cortisol, 0.5 μg/ml) were added and the tubes vortex-mixed. Ethyl acetate (800 μl) was added and the tubes were shaken for 5 min, centrifuged for 1 min and 600 μl of the upper organic phase were removed and added to a 2-ml microcentrifuge tube (hydrolysis tube). A further 600 μl of ethyl acetate were added to the extraction tubes which were then thoroughly vortex-mixed, centrifuged for 1 min, and 600 μl of the upper organic phase were removed and combined with the first extract in the hydrolysis tube. Combined extracts were evaporated to dryness under a gently stream of dry air. To the dried residues, 50 μl of ethanolic sulphuric acid (30:70, v/v) were added. Tubes were vortex-mixed and then incubated at 70°C for 1 min and placed on ice. Water (200 μl) was added, the solutions were vortex-mixed and 800 μl of ethyl acetate were added followed by thorough vortex-mixing. Samples were centrifuged for 1 min, and 600 μl of the upper organic phase were removed and placed into 3-ml polypropylene capped precipitin tubes (Johns Disposable Laboratory Products, Victoria, Australia). A further 600 μl of ethyl acetate were added to the hydrolysis tubes. Tubes were again vortex-mixed, centrifuged for 1 min, and 600 μl of the upper organic phase were again combined with the first extract in the precipitin tube. Combined extracts were evaporated to dryness under a gentle stream of dried air. Dried hydrolysed residues were reconstituted in 100 μl of HPLC mobile phase (60% methanol–40% 5 m*M* triethylamine adjusted to pH 3.3 with orthophosphoric acid).

For the determination of the time course of fluorophore yields, standards (50 ng each of corticosterone and cortisol; 250 ng each of dexamethasone and cortisone) were extracted from 200 μl of water. Optimal excitation and emission wavelengths were determined for corticosterone

and cortisol fluorophores in the HPLC mobile phase using an Aminco SPF500 ratio spectrophotofluorimeter.

Chromatographic procedure

HPLC analyses were carried out using mobile phase prefiltered through a 0.22-μm membrane (Durapore, Millipore, Bedford, MA, USA), with additional gas being removed with an inline Erma Model ERC3522 membrane degassing unit. A Shimadzu LC-6A HPLC pump was used to deliver the mobile phase at 2.0 ml/min, and 20-μl aliquots of samples were applied to a Brownlee Spheri-5 C_{18} column (100 mm × 4.6 mm I.D.), using a Shimadzu Model SIL-9A automatic injector fitted with a 150-μl sample loop. Fluorophores were detected using a Shimadzu RF-535 variable-wavelength fluorescence monitor with excitation and emission wavelengths set at 375 and 485 nm, respectively. Fluorescence data were analysed in terms of peak-area ratios with reference to the internal standard using a Shimadzu CR4-A Chromatopac recording integrator.

RESULTS

The chromatogram of ADX rat serum (Fig. 1A) shows that no peaks corresponding to corticosterone or cortisol were present. A peak of unknown origin associated with the injection front is present in all chromatograms. No interference from gonadal steroids was apparent. Separation of authentic standards is shown in Fig. 1B, where associated minor hydrolysis peaks were acceptably resolved from the major peaks. Typical chromatograms of normal rat serum, with and without added cortisol, are displayed in Fig. 1C and D, respectively. The sample without cortisol gave no response at the retention time of cortisol. For both chromatograms, no additional peaks were seen when compared to ADX serum containing standards (Fig. 1B). Both standards were readily detectable with high sensitivity: corticosterone and cortisol were detectable at 0.1 and 0.25 ng (injected onto HPLC column), respectively. Cortisone, dexamethasone and gonadal steroids were found not to be detectable with the present method.

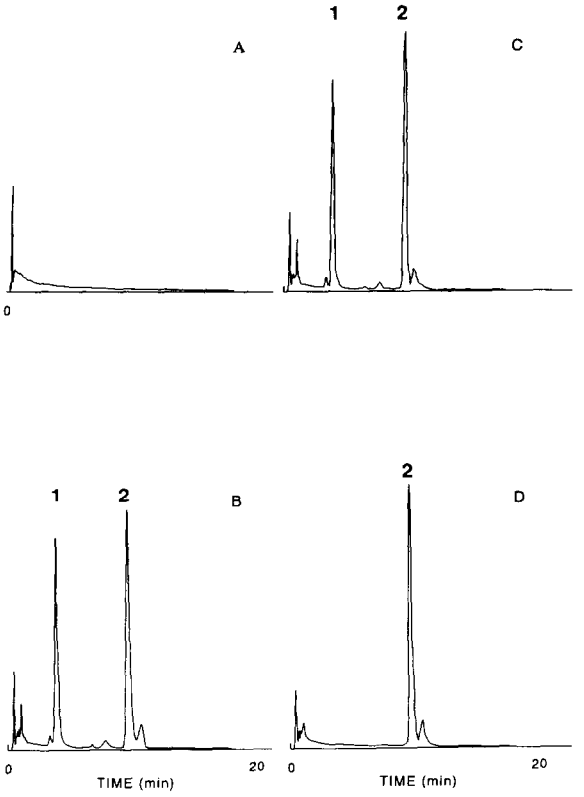

Fig. 1. Chromatograms of rat serum extracts, with and without corticosterone and cortisol. (A) ADX rat serum; (B) ADX rat serum plus authentic standards (corticosterone, 50 ng; cortisol, 50 ng); (C) normal rat serum plus cortisol, 50 ng; (D) normal rat serum. Experimental procedures are described in the text. Extracted, hydrolysed samples were reconstituted in 100 μl of mobile phase with 20 μl being injected onto the HPLC column. Typical retention times were 4 min for cortisol (peak 1) and 10 min for corticosterone (peak 2).

In Fig. 2, the incubation time at 70°C required for optimal fluorophore formation from corticosterone and cortisol is shown. The yield of fluorophore is highly dependent on the incubation time, 1.0 min being chosen as optimal. The recovery (mean ± S.D.) of corticosterone (50 ng) added to ADX and control rat sera was 100.2 ± 0.9 and 98.7 ± 0.8%, respectively (n = 8). A linear relationship for corticosterone concentration (added to ADX serum) *versus* peak area was obtained with a coefficient of variation of 0.999. A similar calibration curve for the ratio of peak areas for corticosterone to cortisol (internal standard) *versus* corticosterone concentration was obtained. The response was linear with a coeffi-

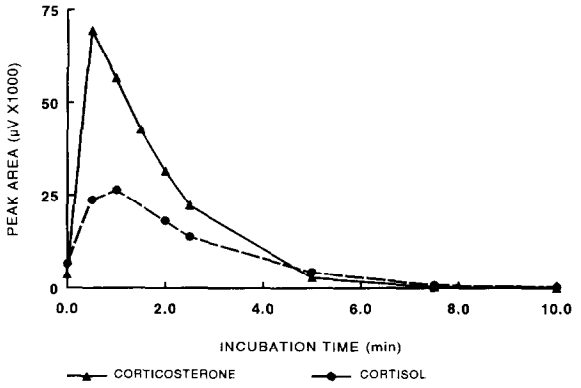

Fig. 2. Incubation time at 70°C required for optimal fluorophore formation from corticosterone and cortisol. Standards (50 ng each) were added to and extracted from water. Experimental procedures are described in the text. Extracted, hydrolysed samples were reconstituted in 100 μl of mobile phase with 20 μl being injected onto the HPLC column. Each point represents the mean of four values.

cient of variation of 0.999. Recovery of corticosterone from water, 5% BSA (a serum substitute) and ADX serum was identical.

The extracted fluorophores were found to be stable for at least fourteen days at ambient room temperature with only 1.5% variation in fluorescence between samples at day 1 compared to day 14 ($n = 6$). Within-day and between-day sample variations were less than 1.7%. Variation in the slopes between replicate calibration curves was less than 2%.

DISCUSSION

A number of organic solvents have been used for extraction of corticosteroids from aqueous samples, in particular methylene chloride [9–13]. Diethyl ether, ethyl acetate and ethanol have been less commonly used. However, in the present work methylene chloride was found to be ineffective for fluorophore extraction following acid hydrolysis. It is also a neurotoxin and a cancer-suspect agent (Mallinckrodt LabGuard guide). Diethyl ether was found to be an effective extractant, but owing to its volatility at ambient temperature, freezing of the lower aqueous phase is required followed by decanting the upper solvent phase. This requires dry ice–acetone freezing mixtures, an unnecessary complication. Ethyl acetate proved to be the method of choice exhibiting reproducible quantitative recoveries of fluorophore. Addition of water after acid hydrolysis was essential for facilitating phase separation although washing of ethyl acetate extracts was not required.

The use of ethyl acetate for steroid extraction has previously been reported [8], however, ammonium carbonate was utilised to help facilitate extraction, an additional step not required in the present method. We have found the ethyl acetate extraction procedure to be highly efficient. It can be quantitatively transferred using autopipettors, forms the upper organic phase (negating the need for aqueous phase removal) and is totally immiscible with water. By using a double extraction (as described in the Experimental section), 94% absolute extraction efficiency is achieved and is highly reproducible. Extraction recovery variability is accounted for by the inclusion of an internal standard. The chromatograms thus obtained have a low background noise (see ADX serum, Fig. 1A). When a recording integrator is used, 0.1 ng (0.29 pmol) of corticosterone fluorophore injected onto the HPLC column is readily detectable.

By the addition of an extraction step after acid hydrolysis, the resultant fluorophores (reconstituted in mobile phase) are stable for at least two weeks at ambient room temperature and two months at −20°C. Samples not extracted after acid hydrolysis require immediate storage at −20°C [9]. The procedure is therefore particularly suitable for use with HPLC systems incorporating automatic sample injectors. This additional extraction step also removes sulphuric acid from the samples which could adversely affect the HPLC column, detector or the sample injector. Post-column hydrolysis systems [10] still have acid entering the HPLC system. Without acid in the system, we have analysed up to 200 samples using only 2 l of mobile phase with recycling. No loss of resolution or variations in retention times were observed. The retention time for the corticosterone fluorophore was typically 10 min. With

TABLE I

CORTICOSTERONE LEVELS OF CONTROL AND LOW-STRESS RATS

Female Wistar rats were allowed voluntary access to running wheels for 25 and 50 days (low-stress animals). Control animals were not exercised. Blood samples were taken at time of sacrifice, 1 h after the start of the light period. Serum corticosterone levels were determined as described in the text. Values shown are means ± S.D. (n = 8). Statistical analyses were made with Student's t-test.

Animal group	Corticosterone level (μg/ml)	
	Control	Low-stress
25 days	0.44 ± 0.12	0.66 ± 0.10[a]
50 days	0.43 ± 0.13	0.56 ± 0.08[a]

[a] $P > 0.05$.

an HPLC run of 15 min, four samples can be analysed per hour.

This procedure is highly specific for both corticosterone and cortisol, with cortisone, synthetic and gonadal steroids/hormones being undetectable and non-interfering. However, compounds such as 11-deoxycorticosterone and 21-deoxycortisol could theoretically be detectable, as these compounds have previously been shown to be detectable with about half the sensitivity shown for cortisol using a post-column hydrolysis method [10].

In our laboratory, the described method is routinely used to check on the efficiency of adrenalectomy of rats. This is especially important since any residual adrenal tissue can regenerate and produce corticosterone. In Table I, serum corticosterone levels for control and exercise-stressed animals are displayed. Values obtained for control animals (at nadir of circadian rhythm) are in agreement with those previously reported using HPLC detection [11,13,18] and radioimmunoassay [1,2]. However, control or resting levels of corticosterone will vary depending on the time of sampling, the season of the year [18], housing conditions and whether the animals are accustomed to being handled. When animals were subjected to low-stress conditions through voluntary access to running wheels, small but significant increases in serum corticosterone levels were found (Table I).

The present method could also be adapted for use with blood, urine or saliva, due to its high sensitivity. Sensitivities can also be varied by altering the volume of sample extracted, the volume of mobile phase used for residue reconstitution or the volume injected onto the HPLC system. With or without the use of an internal standard, calibration standards must be treated similarly as for samples.

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