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Technical note

Rapid and efficient method for extraction and separation of glucocorticosteroids and sex steroids from urines

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Abstract

The influence of different pH values on the recoveries of glucocorticosteroids and sex steroids from Kieselguhr-filled minicolumns has been investigated. While the recoveries of all steroids tested were similar if samples had acidic or neutral pH values, sex steroids could effectively be separated from glucocorticosteroids by increasing the pH value to 13.7: recoveries were 1.7% for glucocorticosteroids and 56–76% for sex steroids. For the determination of sex steroids in biological samples it is recommended to adjust samples to a strong alkaline pH before extraction; this holds especially true for samples with very high glucocorticosteroid levels.

1. Introduction

The classical liquid–liquid extraction of steroids has been replaced by a liquid–liquid extraction in which the aqueous sample is the stationary phase supported by Kieselguhr [1–4]. Using this method we extracted more than fifteen thousand plasma or urine samples of small laboratory animals during the last ten years. While recoveries of steroids were reproducibly high if the pH of the samples ranged between 1 and 7, we recently noticed a diminished recovery of cortisol from urines of adult male guinea pigs. Since most of these samples had extremely variable pH values, we studied the recovery of cortisol and sex steroids as a function of pH.

2. Experimental

2.1. Reagents and solvents

All reagents and solvents (analytical grade) were obtained from Merck (Darmstadt, Germany) or Sigma (Deisenhofen, Germany). Tritiated steroids were supplied by NEN Chemicals (Bad Homburg, Germany). Antisera for steroid assays were generously supplied by Dr. Christine Maser-Gluth (Institute of Pharmacology, University of Heidelberg, Germany).

2.2. Extraction

Experiment 1: 0.1 ml ^{3}H-labelled steroids were added to 0.4 ml 0.5 *M* HCl (pH 0.3), 0.4 ml 0.5 *M*

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Table 1
Influence of pH on the recovery of cortisol and testosterone

Steroid	Recovery (%)						
	pH 0.3	pH 3	pH 5	pH 7	pH 9	pH 12	pH 13.7
Cortisol	78 ± 3[a]	80 ± 2	79 ± 3	76 ± 3	75 ± 4	75 ± 4	3 ± 1
Testosterone	82 ± 5	82 ± 4	80 ± 4	81 ± 4	82 ± 3	77 ± 2	71 ± 4

^{3}H-cortisol and ^{3}H-testosterone, dissolved in 0.5 ml 0.5 *M* HCl, variable portions of 0.5 *M* HCl–0.5 *M* NaOH or in 0.5 *M* NaOH were added to Extrelut minicolumns. Twenty minutes later steroids were extracted with 3.0 ml methylene chloride.
[a] Mean values ± S.D. of 10 determinations.

HCl/0.5 *M* NaOH (pH 3–12) or to 0.5 *M* NaOH (pH 13.7); pH values were checked by a laboratory pH meter. The samples were mixed and applied onto glass pipettes filled with 0.3 g Kieselguhr (Extrelut, Merck). After diffusion into the matrix for 20 min, steroids were extracted with 3.0 ml methylene chloride or diethyl ether. After evaporation of the organic phase, steroids were dissolved in 0.5 ml ethanol and transferred to counting vials. After the addition of 3 ml scintillation fluid the radioactivity was measured in a scintillation counter. Experiment 2: Adult male guinea pigs were intramuscularly injected 0.2 ml saline, (1-24)ACTH (20 IU, Synacthen depot, Ciba Geigy, Wehr, Germany) or HCG (100 IU, Predalon, Organon, Oberschleissheim, Germany). Urine samples were collected between 12 and 24 h after injection. Aliquots of urinary samples were adjusted to pH 13.7, and 0.5-ml aliquots were placed onto Kieselguhr columns; steroids were extracted as described above.

3. Results and discussion

At pH values between 0.3 and 12 about 80% of added cortisol and testosterone were recovered. In contrast, the recovery of cortisol and testosterone differed markedly if pH values were further increased (Table 1). These differences were most obvious if cortisol and testosterone were eluted with diethyl ether (Table 2). Correspondingly, recoveries for other glucocortico-

Table 2
Influence of pH on the recovery of glucocorticosteroids and sex steroids

Steroid	Recovery (%)					
	Methylene chloride			Diethyl ether		
	pH 0.3	pH 7	pH 13.7	pH 0.3	pH 7	pH 13.7
Cortisol	78[a]	78	3	80	81	1
Corticosterone	77	75	7	78	74	3
Testosterone	78	81	71	86	87	72
Progesterone	71	70	56	82	80	63
Androstenedione	85	85	73	94	91	76
Estradiol[a]	22	89	57	23	79	70

^{3}H-steroids, dissolved in 0.5 ml 0.5 *M* HCl (pH 0.3), 0.5 *M* HCl–0.5 *M* NaOH (pH 7) or 0.5 *M* NaOH (pH 13.7) were added to Extrelut minicolumns. Twenty minutes later steroids were extracted with 3.0 ml methylene chloride or diethyl ether.
[a] Mean values of 10 determinations; S.D. values were 0.1–5.5%.

Table 3
Steroid excretion in guinea pigs treated with (1-24)ACTH or HCG

Steroid	Excretion		
	0.9% NaCl	(1-24)ACTH	HCG
Cortisol (μg/12 h)	53 ± 11[a]	234 ± 38	76 ± 24
Testosterone (ng/12 h)[b]	65 ± 7	77 ± 9	150 ± 15
Progesterone (ng/12 h)[b]	12 ± 2	19 ± 2	7 ± 1
Androstenedione (ng/12 h)[b]	983 ± 118	4231 ± 603	1409 ± 252
Estradiol (ng/12 h)[b]	3 ± 1	13 ± 2	5 ± 2

Aliquots of urines of guinea pigs injected with 0.2 ml 0.9% NaCl ($n = 12$), 20 IU (1-24)ACTH ($n = 12$) or 100 IU HCG ($n = 6$) were adjusted to pH 13.7. Samples of 0.5 ml were placed onto Extrelut minicolumns. Twenty minutes later steroids were extracted with 3.0 ml diethyl ether.

[a] Mean values ± standard errors of the means.

[b] In initial experiments samples were adjusted to pH 0.3 before extraction. However, due to the very high cortisol amounts in the samples, values for sex steroids were falsely elevated; therefore, these results were discarded.

steroids (e.g. corticosterone) were low at pH 13.7, while sex steroids were recovered at high percentages.

Taking the large amounts of cortisol in guinea pig urines into account, it is necessary to separate sex steroids from glucocorticosteroids by thin-layer chromatography [5] or high-performance liquid chromatography [3,4]. Since these methods are time-consuming, the procedure described in this note represents an alternative for the separation of major glucocorticosteroids and sex steroids. Its distinct advantages include efficient separation, high reproducibility and low costs of columns and solvents. Furthermore, simultaneous processing of samples enables us to separate sex steroids from glucocorticosteroids in 50–60 urine samples in a single afternoon. The necessity of such separation is shown in Table 3. As is stated in the legend of this table, in initial experiments samples were adjusted to pH 1 before extraction. However, due to the very high cortisol amounts in the samples, values for sex steroids were falsely elevated. However, if samples were brought to pH 13.7 before extraction, we found a significant increase of progesterone, androstenedione and estradiol excretion in guinea pigs treated with 20 IU (1-24)ACTH and increased testosterone and androstendione excretion in animals injected with 100 IU HCG.

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