Journal of Steroid Biochemistry, 1977, Vol. 8, pp. 1105–1108. Pergamon Press. Printed in Great Britain

SIMULTANEOUS DETERMINATION OF THE SULPHATES OF DEHYDROEPIANDROSTERONE AND PREGNENOLONE IN PLASMA BY RADIOIMMUNOASSAY FOLLOWING A RAPID SOLVOLYSIS

T. Aso*, A.-R. Aedo† and S. Z. Cekan‡
Swedish Medical Research Council, Reproductive Endocrinology Research Unit,
Karolinska Hospital, 104 01 Stockholm, Sweden

(*Received* 17 *February* 1977)

SUMMARY

Dehydroepiandrosterone sulphate and pregnenolone sulphate were solvolyzed using hydrochloric acid as catalyst. This procedure was compared with other solvolytic techniques, based on the use of sulphuric acid or acetic acid. The hydrochloric acid procedure exhibited the highest recoveries after 1 h solvolysis. This technique was used for the radioimmunoassay of dehydroepiandrosterone sulphate and pregnenolone sulphate in the plasma of 15 normally menstruating women on 3 selected days of the cycle. Whereas the levels of dehydroepiandrosterone sulphate (approx. 1.5 μg/ml) did not show any changes, there was a significant ($P < 0.001$) difference between the levels of pregnenolone sulphate seen on the day of the LH peak (mean: 57.5 ng/ml) and those found on the 8th day after the LH peak (mean: 102 ng/ml).

INTRODUCTION

A great number of investigations have been reported on the determination of dehydroepiandrosterone sulphate in human blood using gas liquid chromatography or radioimmunoassay, both preceded by an acid-catalyzed solvolysis, e.g., [1–3]. A gas chromatographic method preceded by solvolysis was also used for the measurement of pregnenolone sulphate in plasma [1, 4].

For the solvolysis of steroid sulphates, various techniques have been reported, such as the procedures using the addition of sulphuric acid to urine [5] or plasma [6] and the methods in which sulphuric acid [7, 8] or acetic acid [9] are added to plasma extracts or fractions. In these methods mostly long periods of solvolysis (overnight) were used.

In preliminary attempts to shorten the solvolysis time, hydrochloric acid was employed as acidic catalyst with good results. The aim of the present study was (a) to compare this technique with those described earlier [5–9], as far as the time and yields of solvolysis were concerned, (b) to validate the method selected as a part of a radioimmunoassay procedure by investigating the recognized criteria of reliability [10], and (c) to use it for the radioimmunoassay of a series of human plasma samples.

EXPERIMENTAL

Reagents

All non-conjugated steroids, both labelled and non-labelled, were checked for purity and purified—if needed—according to procedures described in a previous publication [11]. [7-^{3}H]-Dehydroepiandrosterone sulphate ammonium salt (10 Ci/mmol) and [7-^{3}H]-pregnenolone sulphate ammonium salt (20 Ci/mmol) were purchased from New England Nuclear Corp. (Dreieichenhain, Frankfurt/Main, Germany). These compounds were purified by Sephadex LH-20 chromatography. The eluent was chloroform–methanol (1:1, v/v) containing sodium chloride (0.01 mol) [1]. The sulphates were stored in ammoniated ethanol (0.5 ml concentrated ammonia/l 95% ethanol) [12]. Other reagents, materials and assay buffer (0.1 M; pH 7.4) were the same as described previously [11]. The acids used in the present study were purchased from E. Merck, Darmstadt, Germany, and were of analytical purity.

Extraction of unconjugated steroids from plasma

A solution of the radioactive sulphates (approx. 2000 d.p.m./0.1 ml assay buffer each) was added to human blood plasma (20 μl; plasma pool obtained from normally menstruating women) and the vol. was completed to 0.5 ml with assay buffer. The solution was mixed, equilibrated at 40°C for 15 min and the unconjugated steroids were extracted with ethyl ether (5 ml) by vortexing for 1 min. The aqueous layer was frozen in an ethanol–dry ice mixture and the ether solution was decanted and discarded.

Extraction of conjugated steroids by ethyl acetate and solvolysis

Procedure A (modified procedure according to Burstein and Lieberman [5]): The aqueous residue (0.5 ml) was mixed with a saturated solution of

*Ford Foundation Fellow in Reproductive Endocrinology.

† Holder of a World Health Organization Research Training Grant.

‡ To whom requests for reprints should be addressed.

sodium chloride (0.5 ml) and sulphuric acid (0.2 ml; 2 N). This solution was then extracted with ethyl acetate (6 ml twice) by shaking for 10 min. The combined ethyl acetate extracts were kept at 40°C for various periods of time.

Procedure B (modification of the procedure described by Segal *et al.* [7]): The aqueous residue (0.5 ml) was mixed with a saturated solution of sodium chloride (0.5 ml) and extracted with ethyl acetate (6 ml twice) by shaking for 10 min. The combined ethyl acetate extracts were dried with anhydrous sodium sulphate (1 g) and a fresh solution (0.5 ml) of 33 μl concentrated sulphuric acid in 100 ml ethyl acetate was added to the decanted ethyl acetate solution. This was kept at 40°C for various periods of time.

Procedure C (modification of the procedure described by Loriaux *et al.* [9]): The combined ethyl acetate extracts (prepared as described under B) were mixed with glacial acetic acid (1.0 ml). This solution was kept at 40°C for various periods of time.

Procedure D. A drop (approximately 40 μl) of concentrated hydrochloric acid was added to the combined ethyl acetate extracts (cf. procedure B). This solution was kept at 40°C for various periods of time.

Purification of the products of solvolysis and radioimmunoassay

The ethyl acetate solutions were neutralized with a "concentrated carbonate solution" ([13]; 1 ml, pH 10.5), the organic phases were decanted and evaporated at 40°C under nitrogen. The residue was dissolved in assay buffer (1 ml) by heating at 60°C for 10 min, and the solvolyzed steroids were extracted with ether (10 ml). The ether solutions were evaporated under nitrogen and the residues were dissolved in *iso*-octane (1 ml). These solutions were subjected to chromatography on celite columns [11]. Dehydroepiandrosterone and pregnenolone fractions—obtained always from one column—were quantitated using a radioimmunoassay as described previously [11].

Clinical material and calculation of results

Plasma samples used in the present study constituted a portion of the samples collected from 15 normally menstruating women in a previous study [14].

In the calculation of the results of plasma measurements a lognormal distribution of individual values [15] was assumed.

RESULTS

It can be seen from Table 1 that, using procedure A (sulphuric acid added to plasma), the highest recoveries were obtained after 4–16 h of solvolysis. When the solvolysis was performed according to procedure B (sulphuric acid added to the ethyl acetate extract), a high recovery was obtained after 1 h. In the case of pregnenolone sulphate, this recovery almost did not change on prolonged solvolysis. Procedure C (acetic acid added to plasma extract) yielded insufficient recoveries and was not studied further. The solvolysis performed according to procedure D (hydrochloric acid added to plasma extract) gave the highest recoveries after 1 h. These were significantly higher than those obtained by the other methods at that time, and equivalent to those obtained by procedure A after 16 h.

When the procedures A, B and D (C was omitted) were applied to the assay of a plasma pool, using the solvolysis time of 1 h, and when procedural losses occurring in individual measurements were monitored by internal standardization [10, 11], essentially identical results were obtained (Table 2). The procedure D, however, was selected for further studies because of the highest recoveries (cf. Table 1).

Table 1. Recovery* of radioactive steroids following various times of solvolysis and using various solvolytic procedures

	Solvolysis time (h)			
Procedure†	0.5	1	4	16
	Dehydroepiandrosterone sulphate			
A	37.2 ± 4.7	55.7 ± 8.3	65.1 ± 6.8	73.9 ± 4.0
B	33.2 ± 10.3	68.2 ± 9.8	‡	‡
C	2.2 ± 0.8	5.0 ± 2.1	3.9 ± 1.1	10.5 ± 3.1
D	67.1 ± 14.1	78.7 ± 6.7	70.4 ± 7.0	47.6 ± 6.0
	Pregnenolone sulphate			
A	38.7 ± 3.2	58.7 ± 2.8	76.5 ± 10.9	76.7 ± 5.4
B	56.3 ± 3.9	67.3 ± 3.8	66.3 ± 4.6	63.0 ± 15.3
C	2.8 ± 0.8	5.4 ± 3.1	4.0 ± 0.9	10.8 ± 3.5
D	62.5 ± 5.3	72.6 ± 2.3	60.5 ± 9.2	36.2 ± 8.0

* The recovery comprises the yields of the extraction, solvolysis and chromatographic purification. It is expressed as mean percentage of the radioactivity added to a plasma pool ± standard deviation ($n = 10$). The following differences were tested using a *t*-test: dehydroepiandrosterone sulphate: 1 h A vs. 1 h D: $P < 0.001$; 1 h B vs. 1 h D: $P < 0.05$; 1 h D vs. 16 h A: not significant; pregnenolone sulphate: 1 h A vs. 1 h D: $P < 0.001$; 1 h B vs. 1 h D: $P < 0.01$; 1 h D vs. 16 h A: not significant.
† For the meaning of the capital letters cf. Experimental part. ‡ Lost due to a laboratory accident.

Table 2. Steroid sulphate levels* measured in a plasma pool† using 3 different solvolytic procedures (the time of solvolysis was 1 h in all cases)

Procedure	Dehydroepiandrosterone sulphate (ng/ml)	Pregnenolone sulphate (ng/ml)
A	1640 ± 110‡	56.9 ± 4.31
B	1730 ± 100	53.9 ± 3.83
D	1710 ± 116	53.4 ± 4.29

* The levels are expressed in terms of the steroid moieties of the sulphates. No differences between procedures were found using an analysis of variance. † The pool was obtained from 21 normally menstruating women. ‡ Means ± standard deviations; $n = 10$. Individual measurements were corrected for varying recoveries on the basis of internal standard measurements.

Using procedure D and 1 h of solvolysis, the measurements of the within-assay and between-assay variations resulted in coefficients of variation of a generally accepted magnitude (Table 3). The parallelism test [6] did not show any disturbance of specificity.

Measurements of dehydroepiandrosterone, pregnenolone and their sulphates in the plasma of 15 women on 3 selected days of a menstrual cycle indicated (Table 4) that neither dehydroepiandrosterone nor dehydroepiandrosterone sulphate levels exhibited any changes. Pregnenolone and pregnenolone sulphate levels, however, displayed a significant rise ($P < 0.001$) on day 8 following the day of LH peak (day LH + 8) which suggests the occurrence of increased levels during the luteal phase.

DISCUSSION

The time course of solvolysis was different in all 4 methods investigated. The modified method of Burstein and Lieberman [5] (procedure A) showed a slower solvolysis rate than the procedure according to Segal *et al.* [7] (procedure B), in which no major changes in recoveries occurred beyond 1 h of solvolysis. Acetic acid [9] (procedure C) appeared to be a very weak solvolytic agent under the present conditions. It seems that satisfactory recoveries can be obtained only when an overnight solvolysis at elevated temperature (75°C) is performed [3].

The use of hydrochloric acid (procedure D) yielded maximal recoveries after 1 h of solvolysis and the prolongation of the solvolysis time resulted in a marked decrease of recoveries. The decrease of recoveries with the prolonged time of solvolysis must be due to an increased decomposition of the steroids. If, however, any decomposition products (artifacts) are formed during the 1 h of solvolysis, it can be assumed that they are separated on the celite columns and do not enter into the radioimmunoassay proper. This assumption is supported by the test of parallelism which did not detect any measurable amounts of foreign materials in the assay.

The various techniques (except for the solvolysis by acetic acid) were tested in an assay of a plasma pool (1 h solvolysis was used). In this assay the results were corrected for different recoveries (cf. Table 1) on the basis of internal standard measurements in all individual samples. No differences were found as far as final results were concerned. Among the 3 tech-

Table 3. Validation of the radioimmunoassays, using procedure D and 1 h solvolysis

Steroid	Within-assay variation* Mean (ng/ml)	Within-assay variation* C.V (%)	Between-assay variation‡ Mean (ng/ml)	Between-assay variation‡ C.V. (%)	F-value‡ Parallelism	F-value‡ Linearity
Dehydroepiandrosterone sulphate	1650	7.7	1690	15.8	0.01	0.02
Pregnenolone sulphate	53.1	9.3	54.2	14.9	3.62	1.37

* A plasma pool (cf. Table 2) was assayed in 20 replicates. † The pool was assayed on 6 different occasions. ‡ Tabulated $F_{0.95(1,15)} = 4.54$.

Table 4. Geometric means and 95% confidence limits* of the levels (ng/ml) of dehydroepiandrosterone, pregnenolone and their sulphates in plasma in 15 normally menstruating women

Steroid	Cycle day† LH-8	LH	LH + 8	Differences‡
Dehydroepiandrosterone	5.38 (3.90–7.43)	4.90 (3.58–6.79)	4.58 (3.12–6.83)	None
Dehydroepiandrosterone sulphate	1480 (1110–1980)	1570 (1150–2140)	1590 (1150–2190)	None
Pregnenolone	1.33 (1.02–1.74)	1.45 (1.22–1.72)	1.88 (1.70–2.21)	LH − 8 vs. LH: not significant LH vs. LH + 8: $P < 0.05$
Pregnenolone sulphate	70.0 (55.9–89.2)	57.5 (40.0–82.7)	102 (81.5–129)	LH − 8 vs. LH: $P < 0.05$ LH vs. LH + 8: $P < 0.001$

* The figures indicate the concentrations of the steroid moiety of the sulphate. Procedure D and 1 h solvolysis were used for the assays. † LH = samples collected on the day of the LH peak; LH − 8, LH + 8 = samples collected 8 days prior to or 8 days after the LH peak (cf. [14]). ‡ The differences between cycle days were computed using a complete analysis of variance.

niques, that using hydrochloric acid was chosen for the studies of plasma levels. The recoveries were the highest in this procedure and, therefore, the expectation of potential errors in calculation of final results is the least.

The levels of unconjugated steroids found in the present study agree well with the values reported earlier in another group of subjects [16]. The levels of dehydroepiandrosterone sulphate and pregnenolone sulphate are close to those described by other investigators, e.g., [17, 18, 1].

The sulphates seem to follow the menstrual pattern of their unconjugated counterparts, i.e., neither dehydroepiandrosterone [16] nor dehydroepiandrosterone sulphate levels seem to change during the cycle, and both pregnenolone [16] and pregnenolone sulphate seem to exhibit increased levels in the luteal phase. These conclusions, however, remain to be confirmed by analyzing the levels of the sulphates on all days of the menstrual cycle.

Acknowledgements—The expenses of this investigation were defrayed by research grants from the Swedish Medical Research Council, the Ford Foundation and the World Health Organization.

REFERENCES

1. Jänne O., Vihko R., Sjövall J. and Sjövall K.: *Clin. chim. Acta* **23** (1969) 405–412.
2. Sekihara H., Ohsawa N. and Ibayashi H.: *Steroids* **20** (1972) 813–824.
3. Nieschlag E., Loriaux D. L. and Lipsett M. B.: *Steroids* **19** (1972) 669–679.
4. Scommegna A. and Bieniarz J.: *J. clin. Endocr. Metab.* **33** (1971) 787–792.
5. Burstein S. and Lieberman S.: *J. biol. Chem.* **233** (1958) 331–335.
6. Shutt D. A., Smith I. D. and Shearman R. P.: *J. Endocr.* **60** (1974) 333–341.
7. Segal L., Segal B. and Nes W. R.: *J. biol. Chem.* **235** (1960) 3108–3111.
8. Vihko R.: *Acta endocr., Copenh. suppl.* **109** (1966) 1–69.
9. Loriaux D. L., Ruder H. J. and Lipsett M. B.: *Steroids* **18** (1971) 463–472.
10. Cekan S. Z.: *Acta Univ. upsalien.* No. **14** (1976) 1–48.
11. Brenner P. F., Guerrero R., Cekan Z. and Diczfalusy E.: *Steroids* **22** (1973) 775–794.
12. Rosenthal H. E., Pietrzak E., Slaunwhite W. R. Jr. and Sandberg A. A.: *J. clin. Endocr.* **34** (1972) 805–813.
13. Brown J. B.: *Biochem. J.* **60** (1955) 185–193.
14. Aedo A.-R., Landgren B.-M., Cekan Z. and Diczfalusy E.: *Acta endocr., Copenh.* **82** (1976) 600–616.
15. Gaddum J. H.: *Nature, Lond.* **156** (1945) 463–466.
16. Guerrero R., Aso T., Brenner P. F., Cekan Z., Landgren B.-M., Hagenfeldt K. and Diczfalusy E.: *Acta endocr., Copenh.* **81** (1976) 133–149.
17. Buster J. E. and Abraham G. E.: *Analyt. Lett.* **5** (1972) 543–551.
18. Metcalf M. G.: *Steroids* **28** (1976) 311–324.