

## Technical methods

### Simple method for the isolation of 17-oxosteroid glucuronides from urine

D. GUPTA *From the Institute of Child Health, Department of Growth and Development, University of London*

In order to study the hydrolysis, enzymatic or chemical, of the plasma and urinary steroid glucuronides, as well as the biosynthesis, the isolation of the individual 17-oxosteroids is an absolute necessity. Schneider and Lewbart (1959) employed counter-current distribution techniques to isolate several steroid glucuronides. Androsterone glucuronide was isolated by Edwards and Kellie (1956) and by Crepy, Jayle, and Meslin (1957); and the aetiocholanolone salt was isolated by Baulieu (1960). Hadd and Dorfman (1963) isolated androsterone glucuronide from a patient producing a massive amount of it. Since then both 5 $\alpha$  (Staib and Doenges, 1960) and 5 $\beta$  (Staib and Doenges, 1960; Hadd, 1958) forms of the 17-oxosteroid glucuronide have been synthesized. The object of the present work was to develop a method of separating the major 17-oxosteroid glucuronides on a large scale and economically. In the techniques developed inexpensive and easily available apparatus is used.

#### METHODS AND RESULTS

The method consists of the following steps:

**1 ADMINISTRATION OF PURE STEROID** Pure androsterone (Organon Laboratories), 9 g., dissolved in absolute alcohol, was taken orally by a normal adult male, divided in nine portions over a period of three days. Urine specimens were collected before, during, and 48 hours after the dose was discontinued. The normal 17-oxosteroid level returned within a period of 48 hours to the pre-administered level. About 45% of the administered steroid was recovered as 17-oxosteroid conjugates. Paper chromatographic results showed that all the excreted steroids were in the conjugated form.

#### **2 EXTRACTION OF THE STEROID CONJUGATES FROM URINE**

All the collected urine specimens were pooled together and extracted in liquid-liquid extractor with methylene chloride after adjusting the urine to pH 3. The total extract was dried, dissolved in ethanol, and divided into four suitable portions.

**3 CHROMATOGRAPHY ON SILICA GEL** Each portion was dried and chromatographed separately on silica gel column (length 30 cm.; diameter 3 cm.) with 0.1N sodium

acetate (0.6 ml./g. of silica gel) as the stationary phase. Elution was effected with chloroform containing 2% (v/v) ethanol and 0.7% (v/v) acetic acid. Fractions, each of 10 ml., were collected. Each fraction was checked for the Zimmermann reaction (1935) for 17-oxosteroids and by the naphthoresorcinol test (Kamil, Smith, and Williams, 1953) for the glucuronide salt.

**4 CHROMATOGRAPHY ON CELITE-535** The fractions which gave positive Zimmermann as well as naphthoresorcinol tests were pooled and run again on a Celite-535 partition column (length 30 cm.; diameter 3 cm.) with benzene:methanol:ethyl acetate:water:acetic acid: 350:150:100:350:50. Fractions, each of 10 ml., were collected and analysed as before. There was sharp and satisfactory resolution between the glucuronides of androsterone, aetiocholanolone, 11-hydroxyaetiocholanolone, 11-hydroxyandrosterone, 11-oxoaetiocholanolone, and 11-oxoandrosterone. Except androsterone (the administered steroid) all the other steroid glucuronides were of much smaller quantities.

**5 PURIFICATION ON SEPHADEX** Further purification of the individual 17-oxosteroid glucuronides obtained was carried on a 10 cm. Sephadex G-50 column (Gupta, 1963) using 50% methanol as the eluting fluid.

Portions of the individual steroid glucuronides were checked for purity by the naphthoresorcinol test for glucuronide, by the Zimmermann reaction for 17-oxosteroids, running samples on paper chromatographs (Gupta and Tanner, 1965) to check whether there were any free steroids, hydrolysis by  $\beta$ -glucuronidase and paper chromatography of the liberated steroids after hydrolysis, and comparing the acetates, formates, propionates, and benzoates of the hydrolyzed steroids with these derivatives made from authentic steroids.

**6 S-BENZYLTHIURONIUM SALT OF 17-OXOSTEROID GLUCURONIDES** Androsterone glucuronide was finally crystallized as its salt of S-benzylthiuronium chloride following the method described by Vogel (1948). Recrystallization was carried out from hot water.

#### DISCUSSION

It was observed that the amount of androsterone ultimately excreted as sulphate was uninfluenced when the administered dose was low, but increasing the dose considerably resulted in a steep rise in the amount of androsterone ultimately excreted as sulphate. This agreed well with the observations made by Schneider and Lewbart (1959). It was also observed that during the initial stage of administration the glucuronide excretion exceeded that of the sulphate but when the dose was stopped the sulphates dominated the excretion pattern. The explana-

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## Improved method for estimating blood sugar

NIYAM CHARAN SHARMA AND BIMAL KUMAR SUR  
From the Department of Physiology, G.S.V.M.  
Medical College, Kanpur (India)

True blood-sugar estimation is performed routinely by the Asatoor and King method (1954) in a large number of hospital laboratories. Their method is a colorimetric modification of one (Harding and Downs, 1933) of the most accurate iodometric titration procedures, in which

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tion of this may be that the renal clearance of the sulphates is a much slower process (Bongiovanni, 1958).

As anticipated, the major part of the recovered 17-oxosteroids was accounted for as androsterone glucuronide, but a large amount was also conjugated with sulphuric acid. Schneider and Lewbart (1959) found that only a small amount of administered androsterone was converted to isoandrosterone but in this experiment a large proportion of the administered androsterone was converted to isoandrosterone. This steroid was solely conjugated with sulphuric acid and its percentage excretion was fairly constant and was not dependent on the total output of the steroid. We failed to detect in either the glucuronide or the sulphate fraction any androstan-3,17-dione, which is the metabolic intermediate between androsterone and isoandrosterone. There was also no conversion to aetiocholanolone.

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reduced copper is oxidized to cupric ions by means of iodine. If no necessary allowance has been made, the cupric ions formed would be completely reduced and precipitated as cuprous iodide in the presence of an excess of soluble iodide in the reagent. Probably to check this reduction by iodide, Harding and Downs (1933) employed potassium oxalate in their alkaline reagent because oxalate inhibits more or less completely the reaction of the cupric salt with soluble iodides by forming alkali cuprioxalate which dissociates to give cuprioxalate anions (Elbs, 1917; Shaffer and Hartmann, 1921; DeLong, 1927). Asatoor and King (1954), without considering the fundamental role of potassium oxalate, retained it in the alkaline reagent in their colorimetric modification of the method which makes no use of iodometry. It appears that potassium oxalate plays no role in the colorimetric method and, therefore, we modified the method of Asatoor and King by omitting it in their alkaline reagent.

### MATERIALS AND METHODS

In addition to the reagents used by Asatoor and King (1954), a new modified alkaline reagent containing 25 g.  $\text{NaHCO}_3$ , 20 g.  $\text{Na}_2\text{CO}_3$ , and 12 g. Rochelle salt ( $\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$ ) per litre has also been introduced.

For estimation of glucose in standard solutions, the method described by Asatoor and King (1954) for true blood sugar determination, was used in its original form and in the form utilizing the new alkaline reagent. The optical densities of the blue solutions obtained with phosphomolybdic acid reagent were read on a Klett-Summerson photoelectric colorimeter using a 66-red filter. The strength of glucose solutions used has been expressed as corresponding to milligrams per 100 ml. blood and not as its actual concentration.

### RESULTS AND DISCUSSION

Figure 1 shows that the blue colour formed by the Asatoor and King method obeys Beer's law for glucose solutions of concentrations above 100 mg. per 100 ml. and not below this strength. In the range 40 to 80 mg. glucose per 100 ml., values obtained are lowered to the extent of 14 to 32%. Apparently for this reason, Asatoor and King introduced a second glucose standard (corresponding to 80 mg. per 100 ml. blood) in their method. A fasting normal blood sugar value of around 60 mg. per 100 ml. would be about 15% too low even if a glucose standard of 80 mg. per 100 ml. is used. Correct values in this region can, however, be obtained only if the readings of the test and standard are close to each other. While using this method, we frequently observed low normal blood-sugar values and it appears from the results (Fig. 1) that the lack of linearity in this region might be responsible for low normal blood-sugar values.

In the improved method omitting potassium oxalate, the optical density recorded was proportional to the concentration of glucose even in solutions as dilute as 20 mg. per 100 ml. (Fig. 1). Correct values for blood sugar can, therefore, be obtained using a single glucose standard.

Dilution of a stock molybdenum blue solution shows