

# The Detection and Determination of Medicinally Important Steroidal Compounds

By

DR. EFRAIM NJAU,  
Department of Pharmacy, Medicinal Chemistry Section, University of Aston in Birmingham, Gosta Green, Birmingham B4 7ET, U.K.

### SUMMARY

The development of a method for the detection and possible determination of steroidal substances of medicinal importance is reported. Para-phenylenediamine in acetic acid is shown to be specific for locating a B-unsaturated keto-steroids, while Chloramine-T and trichloroacetic acid can be used as general purpose reagent for detection of steroidal substances on thin layer plates.

Amongst the steroids are substances of very considerable medicinal importance. These include sex hormones such as oestrone, androsterone and progesterone, the adrenocortical hormones such as cortisone, and the antiarachitic vitamin - Vitamin D<sub>2</sub> and the cardiac glycosides e.g. digoxin which is a sugar derivative of the steroid digoxigenin. Also marketed is the steroidal antibiotic, fusidic acid. Related to these compounds are a wide spectrum of analogues which have been introduced into therapy to correct hormone deficiencies, as anabolic agents, to alleviate rheumatoid arthritis, various dermatological conditions and even more widely as oral contraceptives. Bile-acids occur as conjugates with amino-acids. Thus glycocholic acid, RCONH.CH<sub>2</sub>.CO<sub>2</sub>H contains a steroid fragment, cholic acid linked as an amide to

glycine. The sodium salts of these conjugates play the important role of facilitating absorption of fat from the intestine. The detection and determination of these substances is of obvious pharmaceutical and medical interest.

From the number of methods described for the analysis of steroids it can be safely assumed that these compounds represent an important and difficult to determine class of substances. A review in 1969 (Lisboa) quotes over eighty colorimetric agents for the detection of steroidal compounds.

As part of a programme into the development of sensitive and if possible specific luminescence methods for the determination of steroidal substances in biological fluids work has commenced on the evaluation of some of the colorimetric agents for this purpose. It was found that an assessment of the reagent's potential for quantitative analysis could be obtained by using it as a spray on the compounds on thin layer chromatography (T. L.C.) plates. Examination of the produced fluorescence gave a rapid indication of the reagent's specificity and approximate sensitivity.

TABLE 1

| STEROIDAL COMPOUND                                                                                                                                                            | REAGENT                   |     |                       |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------|-----|-----------------------|-----|
|                                                                                                                                                                               | 1% Aniline in 25% aqueous |     | 1% PPD in 25% aqueous |     |
|                                                                                                                                                                               | HOAC                      | TCA | HOAC                  | TCA |
| <i>Applied at 0.4µg on to T.L. silica plate, sprayed with reagent, baked for 5min. at 105°, visualised under 350nm., (+)ve signs indicate visual estimate of fluorescence</i> |                           |     |                       |     |
| 1. 5α-Androstan-17β-o1, 3-one                                                                                                                                                 | —                         | —   | —                     | —   |
| 2. 5α-Androstan-17α-methyl-3β, 17β-diol                                                                                                                                       | —                         | +   | —                     | ++  |
| 3. 5α-Androstan-3α-o1, 17-one (Androsterone)                                                                                                                                  | —                         | —   | —                     | —   |
| 4. 5α-Androstan 3β, 17β-diol.                                                                                                                                                 | —                         | —   | —                     | —   |
| 5. 5-Androsten 3β-o1, 17-one (Dehydroepiandrosterone)                                                                                                                         | —                         | +   | —                     | ++  |
| 6. 4-Androsten 3, 17-dione                                                                                                                                                    | —                         | —   | ++                    | —   |
| 7. 4-Androsten 3, 11, 17-trione                                                                                                                                               | —                         | —   | ++                    | —   |
| 8. 4-Androsten-17β-o1, 3-one (Testosterone)                                                                                                                                   | —                         | —   | ++                    | —   |
| 9. 4-Androsten-17α methyl, 17β-o1, 3-one                                                                                                                                      | ++                        | +++ | ++                    | ++  |
| 10. 5-Androsten-17α-methyl, 3β, 17β-diol                                                                                                                                      | —                         | +   | —                     | —   |
| 11. 5α-Pregnan 3, 20-dione                                                                                                                                                    | —                         | —   | —                     | —   |
| 12. 5α-Pregnan 3β-o1 20-one                                                                                                                                                   | —                         | —   | —                     | —   |
| 13. 5β-Pregnan 3α, 20α-diol. (Pregnanediol)                                                                                                                                   | —                         | —   | —                     | +   |
| 14. 5β-Pregnan-3α, 6α-diol, 20-one                                                                                                                                            | —                         | +   | —                     | —   |
| 15. 4-Pregnen-3, 20-dione (Progesterone)                                                                                                                                      | —                         | —   | ++                    | —   |
| 16. 4-Pregnen-17α, 21-diol 3, 11, 20-trione (Cortisone)                                                                                                                       | ++                        | +   | ++                    | —   |
| 17. 4-Pregnen-11α-o1, 3, 20-dione                                                                                                                                             | —                         | —   | ++                    | —   |
| 18. 4-Pregnen-21-o1-3, 20-dione                                                                                                                                               | —                         | —   | ++                    | —   |
| 19. 4-Pregnen-3, 11, 20-trione                                                                                                                                                | —                         | +   | ++                    | —   |
| 20. 5-Pregnen-3β, 20β diol.                                                                                                                                                   | —                         | +   | —                     | ++  |
| 21. 5-Pregnen-3β, 17α-diol; 20-one                                                                                                                                            | —                         | +   | —                     | ++  |
| 22. 5-Pregnen-3β-o1, 20-one                                                                                                                                                   | —                         | +   | —                     | ++  |
| 23. Digoxin                                                                                                                                                                   | —                         | ++  | —                     | ++  |
| 24. Digitoxin                                                                                                                                                                 | ++                        | ++  | —                     | ++  |
| 25. Gitoxin                                                                                                                                                                   | ++                        | ++  | —                     | ++  |
| 26. Ouabain                                                                                                                                                                   | —                         | —   | —                     | —   |
| 27. Cholesterol                                                                                                                                                               | —                         | +   | —                     | ++  |

No reaction was obtained with o- and m phenylenediamines-in either HOAC or TCA.

Fluorescent spots  
Fluorescent spots  
Non fluorescent spots



TABLE 2

Steroid numbered as in Table 1 REAGENT sprayed onto steroid at 5-10 $\mu$ g on T.L. silica plate, then baked at 120 $^{\circ}$  for 5 min. and visualised under 350nm. \*Substances not detected below 0.4 $\mu$ g level.

|     | HOAc | HOAc/CLAT | MCA | MCA/CLAT | DCA | DCA/CLAT | TCA | TCA/CLAT |
|-----|------|-----------|-----|----------|-----|----------|-----|----------|
| 1.  | —    | —         | —   | —        | —   | —        | —   | —        |
| 2.  | —    | ++        | +   | +++      | +   | +++      | +   | +++      |
| 3.  | —    | —         | —   | —        | —   | —        | —   | —        |
| 4.  | —    | —         | —   | —        | —   | —        | —   | —        |
| 5.  | —    | +++       | —   | +++      | —   | +++      | ++  | +++      |
| 6.  | —    | +++       | —   | +++      | —   | +++      | —   | +++*     |
| 7.  | —    | —         | —   | —        | —   | —        | —   | —        |
| 8.  | —    | +++       | —   | +++      | —   | +++      | +   | +++*     |
| 9.  | +++  | +++       | +++ | +++      | +++ | +++      | +++ | +++      |
| 10. | +++  | +++       | +++ | +++      | +++ | +++      | +++ | +++      |
| 11. | —    | —         | —   | —        | —   | —        | —   | —        |
| 12. | —    | —         | —   | —        | —   | —        | —   | —        |
| 13. | —    | —         | —   | —        | —   | —        | —   | +++      |
| 14. | —    | —         | —   | —        | —   | —        | —   | —        |
| 15. | —    | +         | —   | ++       | —   | +++      | —   | +++      |
| 16. | —    | —         | —   | —        | —   | —        | —   | —        |
| 17. | —    | —         | —   | —        | —   | —        | —   | —        |
| 18. | +    | ++        | +   | +++      | —   | +++      | +   | +++*     |
| 19. | +    | +         | +   | +        | +   | +        | +   | +        |
| 20. | +    | ++        | +   | ++       | +   | +        | +   | ++       |
| 21. | —    | —         | +   | +        | +   | +        | +   | ++       |
| 22. | —    | +++       | —   | +++      | +   | +++      | +   | +++      |
| 23. | +    | +++       | —   | +++      | +++ | +++      | +   | +++      |
| 24. | ++   | +++       | ++  | +++      | ++  | +++      | +   | +++      |
| 25. | ++   | +++       | ++  | +++      | ++  | +++      | +++ | +++      |
| 26. | —    | +         | +   | ++       | —   | +++      | —   | +++*     |
| 27. | +    | ++        | +   | ++       | ++  | +++      | ++  | +++      |

Prompted by the report that  $\alpha$ -hydroxyketones can be detected by reagents containing m-, and p-phenylenediamines in acetic acid (Gray and Wright, 1965) these amines and also aniline and o-phenylenediamine were used for the detection of steroidal compounds. It was considered that these reagents might be reactive with the corticosteroids. Table 1, summarises the results obtained by spraying over twenty steroidal substances with the amines in either acetic acid (HOAc) or trichloroacetic acid (TCA). With the steroids at 0.4 $\mu$ g levels only aniline and p-phenylenediamine (PPD) in HOAc produced detectable chromophores. Aniline developed fluorescent spots with four of the compounds and PPD gave rise to non-fluorescent brown spots with nine of the compounds. The reaction of PPD is seen to be structure specific. The colour is obtained only with those steroids which have an unsaturated conjugated carbonyl 4-en-3-one, system in ring A. On using the same amines in TCA the specificity is lost and both aniline and PPD detect roughly the same 10-13 steroids. On attempting to apply the HOAc system to the estimation of the steroids in solution it was found that both the fluorescence produced with aniline and the absorption with P.P.D. was not quantitative and insensitive at levels below 1 $\mu$ g of steroid. It has been suggested (Frerejacques and Graeve, 1963) that a mixture of chloramine-T, (CLAT) in TCA produces stable fluorescent products with cardiac glycosides on TLC plates. The use of this reagent for the detection of other steroids or for the estimation of either group of compounds in solution has not been reported. We have examined the scope of this reagent and the results are summarised in Table 1. The role of the acid was determined by a prior examination of the effect of TCA, HOAc, mono- and dichloroacetic acids, (MCA and DCA), in the absence of CLAT. It can be seen, Table 2, that all four acids produce fluorescence in the absence of CLAT with many of the steroids. The TCA reacts with more of the compounds than the other three acids. The presence of CLAT with acids, particularly with TCA, appears to increase the range of detection in terms of the number of compounds which react. The results show that a mixture of TCA/CLAT

may be used as a general purpose spray for the detection of steroidal compounds at 5-10 $\mu$ g levels. At levels of 1-5 $\mu$ g the number of steroids detected is reduced by four and below these levels intense fluorescence is observed from only the cardiac glycosides, digoxin, digitoxin and gitoxin. With the exception of 4-androsten-17 $\alpha$ -methyl, 17 $\beta$ - $\alpha$ 1, 3-one, only six other steroids show a weak fluorescence at this level.

Since the cardiac glycosides are biologically important and the number of sensitive and specific assay methods is small it was decided to develop the reaction of TCA/CLAT for their determination in solution. Initial work with digoxin showed that there was a good linear reaction between concentration and fluorescence intensity from 0.02 to 1.6 $\mu$ g/ml. The fluorophore appears to be formed readily and the above range is not the lowest limit of detection. The optimum conditions for this determination and its specificity were recently reported (Britten and Njau, 1975).

In conclusion this paper proposes that a solution of p-phenylene-diamine in acetic acid can be used as a spray reagent for locating  $\alpha$ -B-unsaturated steroidal ketones without interference from other steroids and that a mixture of Chloramine-T and trichloroacetic acid can be used as a general purpose reagent for the location of steroidal substances on TLC plates.

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