QUALITATIVE, QUANTITATIVE AND PREPARATIVE CHROMATOGRAPHY OF STEROIDS ON FULLY ACETYLATED PAPER

II. QUANTITATIVE CHROMATOGRAPHY OF Δ^4- AND $\Delta^{1,4}$-3-KETOSTEROIDS*

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INTRODUCTION

Various methods are known for quantitative paper chromatography of weakly polar steroids. Use is often made of impregnated paper in order to ensure proper separation, although this involves drawbacks of various kinds. Upon elution of the spots after chromatography, the solution to be measured also often contains the impregnating substance, which may adversely affect spectrophotometric determination through causing high, irregular paper blanks. As these impregnating substances are often nonvolatile, they are difficult to remove[1]. Also in colorimetry, in which steroids are converted into coloured compounds, the impregnating substance may have a disturbing effect.

If untreated paper is used for chromatographic separation, adsorption of steroids on the paper may cause substantial losses during development of the chromatogram and during elution of the spots[2].

These difficulties can be obviated by using fully acetylated paper. A method of qualitative separation on this paper has already been developed by us[3] and in a preliminary communication we stated that this method could also be used for quantitative and preparative purposes[4]. The present paper describes the quantitative determination more fully. Owing to there being no impregnating agent and because steroids are virtually not adsorbed on acetylated paper, a high degree of accuracy was obtained.

METHODS

A. Papers

A strip of untreated Whatman No. 4 paper, 7 × 17 cm, was sewn, with a 1 cm overlap, to a sheet of Whatman No. 1 paper, 44 × 17 cm, fully acetylated in our laboratory (ZIJP's method[5], slightly modified[3]), in order to retard the mobile phase.

The paper was then chromatographically washed by the descending method

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with 100 ml methanol p.a.* and afterwards dried for 10 min at 80°. A strip 1.5 cm wide was cut from both long sides of the paper, as impurities often remain in the edges. The length of the retardation strip was then decreased 4 cm. The starting line was marked 5 cm from the bottom of this strip, *i.e.* 3 cm from the bottom of the acetylated paper. The sheet was then divided into five "runs" with pencil lines.

B. Application of the steroids to the paper

On the starting line two spots were applied of a solution of the steroid mixture for investigation and two spots of a standard solution of the steroid to be determined.

The aim was a dosage of 80–120 μg of the steroid in 80 μl of methanol. If the quantity of steroid in the mixture was not known approximately, a trial chromatogram was made first. For this purpose varying quantities of a solution of the mixture were employed beside a known quantity of the steroid to be determined. After chromatography the spots were compared and the concentration of the steroid in the solution of the mixture was estimated.

Dosing was done by means of an Agla micrometer syringe** in sixteen fractions of 5 μl each, which were meanwhile blown dry with nitrogen in order to keep the spots small. One of the five runs on the sheet was left open for determination of the paper blank (see D).

C. Chromatography

After application of the spots, the acetylated paper was hung in a chromatography cylinder (19 cm in diam.; 50 cm in height) and accommodated for 16 h at 23° over 120 ml of the lower layer (with high benzene content) of the liquid mixture: benzene p.a.–methanol p.a.–water (4:4:1). Next the chromatogram was developed for 8 h with the upper layer (with high methanol content) by the ascending technique (method B, see[1]). The chromatogram was then dried at 60° for 5 min.

The spots can be marked under a "Chromatolite" lamp*** (max. 253.7 mμ), as they are visible as dark spots in U.V. light of this wavelength. In the brief time required for this marking, no photochemical conversion or attack could be found.

D. Elution

The slightly oval spots were cut out with a small margin so that one of the short sides became pointed and the other was folded over for a length of about 3 mm. This edge was held between two glass slides placed at an incline in a porcelain dish filled with methanol p.a. (DENT'S elution method[6]). As the acetylated paper is fairly thick, the distance between the two slides was too great for adequate capillary attraction of methanol. By grinding one of the slides at the place where the paper lies, however, the space between the slides was made small enough. The strip of paper hung point

* Methanol pro analysi, und für die Chromatographie. Merck.
** Burroughs Wellcome Co., London.
*** Hanovia Ltd., Slough, England.

downwards over a special 10 ml calibrated, pear-shaped distillation flask; 7 to 8 ml eluate was collected. (It was found experimentally that a spot of 100 μg cholestenone with 7 ml methanol could be 100% eluted from the acetylated paper. A similar test with non-acetylated Whatman No. 1 paper, however, showed an 8% loss.)

A similar piece of paper was cut from the control run at the same height as the spot being determined and was likewise eluted. The eluate may contain traces of

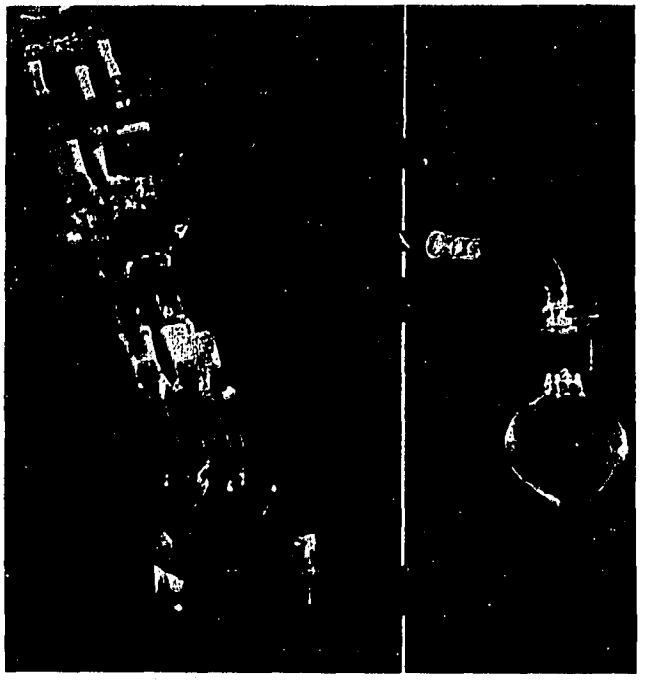

Fig. 1. Apparatus for combined distillation from three calibrated measuring flasks.

benzene from the chromatogram. As benzene disturbs spectrophotometric determination it was removed by evaporation in vacuum under nitrogen. A simple unit was built to which three measuring flasks can be connected at once (Fig. 1). The residue was made up to 10 ml with methanol p.a.

E. Spectrophotometric determination

Before the solutions obtained were measured in a spectrophotometer*, the calibration curve of the steroid was determined by measuring two methanolic solutions of the steroid in various dilutions against methanol. Quartz glass cuvettes** were used, while measurement took place at the $\lambda_{max.}$ of the steroid. For cholestenone we found 242 mμ, for progesterone 240.5 mμ, for testosterone 241 mμ, for hydrocortisone 241.5 mμ and for prednisolone 243 mμ.

The extinction values were then determined for the solutions obtained after chromatography and elution. The value found for the paper blank was deducted.

Losses that might arise through chromatography were determined by measuring

* Unicam spectrophotometer SP 500.
** Quartz glass Ultrasil.

the chromatographed and eluted standard spots. These losses were found to be approximately proportional to the quantities applied (Fig. 2).

To find any possible error in dosage, the same volume of the standard solution that was applied to the paper, was also added to a measuring flask with the aid of the same syringe and was subsequently made up to 10 ml with methanol. By determining

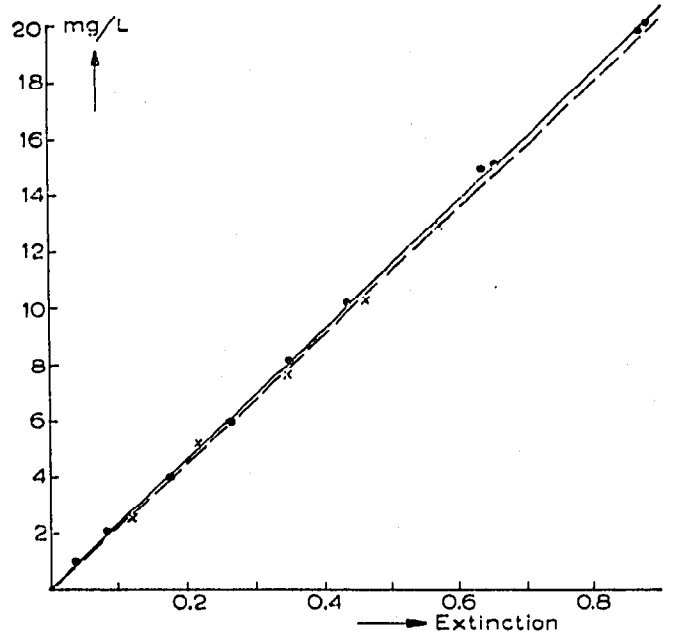

Fig. 2. Cholestenone extinctions determined at 242 mμ: —— calibration curve obtained with standard solution (• •); – – – calibration curve obtained with chromatographed standard (× ×).

the extinction of this solution, it was ascertained via the calibration curve how much steroid had really been applied to the paper.

The corrected extinction value of the steroid that is to be determined can then be calculated with the formula:

$$a = \frac{c}{b - d}(e - d)$$

in which: a = corrected extinction of the steroid,
b = measured extinction of the solution of the steroid chromatographed and eluted from the standard solution,
c = measured extinction of solution of a similar quantity of steroïd not chromatographed,
d = measured extinction of solution obtained after elution of the paper blank from the control strip,
e = measured extinction of solution of the steroid to be determined after chromatography and elution.

With the aid of the calibration curve and value a the steroid concentration can be ascertained.

RESULTS

The method was tried out with the following steroids: Δ^4-cholesten-3-one, progesterone, testosterone, hydrocortisone and prednisolone.

The accuracy of the method was ascertained for cholestenone and progesterone by means of twenty determinations. For each of these substances four strips of paper were used as described in A. Per sheet, five spots were applied in quantities of 26, 52, 78, 104, 130 μg and 25, 49, 74, 99 and 124 μg, respectively. Chromatography and elution were carried out by the method described and the concentration of the solutions was determined spectrophotometrically.

The results show that with a dosage of approx. 25 μg the errors are greatest. Spots of 50–125 μg had about the same relative standard deviation. With sixteen determinations (50–125 μg) it was 4.0% for cholestenone and 3.8% for progesterone.

For testosterone a total of nine determinations were made, *i.e.* in dosages of 49 μg (three), 73 μg (three) and 97 μg (three). The relative standard deviation in this case was 4.9%.

Random tests with hydrocortisone and prednisolone also indicated that these steroids can be determined by the method described.

Lastly, this method was tested with two trial mixtures of cholestenone and

TABLE I

	Extinction (av.)	*Quantity*	*Percentage of steroid in mixture*	
			as found	*actual*
Mixture I				
Cholestenone determination				
Paper blank	0.038			
Stand. soln.	0.428			
Stand. soln. after chrom.	0.438			
134 μg mixture	0.436	99 μg	74	73
Progesterone determination				
Paper blank	0.038			
Stand. soln.	0.524			
Stand. soln. after chrom.	0.514			
309 μg mixture	0.458	86 μg	28	27
Mixture II				
Cholestenone determination				
Paper blank	0.050			
Stand. soln.	0.428			
Stand. soln. after chrom.	0.460			
324 μg mixture	0.331	68 μg	21	22
Progesterone determination				
Paper blank	0.038			
Stand. soln.	0.524			
Stand. soln. after chrom.	0.570			
141 μg mixture	0.633	109 μg	77	78

progesterone. Two paper strips were used for each mixture, one for cholestenone and one for progesterone. 20.628 mg of mixture I and 21.635 mg of mixture II were each dissolved in 10 ml methanol.

The results are given in Table I.

The absolute error in the determined percentage of the steroid in the mixture in all four cases was found to be only about 1%.

DISCUSSION

Chromatographic washing of acetylated paper with methanol proved to be necessary in order to obtain low and only slightly varying blanks.

During dosage of the steroids, losses may occur through the solution creeping along the syringe. By grinding the needle square and holding it at an angle to the paper during application of the solution, the loss can be kept very low and virtually constant.

The chromatogram is dried at low temperature to obviate any losses through atmospheric oxidation.

The elution method employed is more satisfactory than that with which the paper strips are shaken in solvent[1], as this leaves fine paper fibres floating in the solution.

The presence of benzene in the eluate causes high and greatly varying control values owing to the steep absorption peaks of benzene in the measuring range. As intensive drying of the paper did not suffice to remove the benzene quantitatively, the eluate was dried by evaporation *in vacuo*.

Use of the same combined distilling and measuring flask for collecting and evaporating the eluate and subsequent dissolving in methanol in order to determine the extinction obviated decanting losses.

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SUMMARY

A method has been developed for quantitative chromatography of Δ^4- and $\Delta^{1,4}$-3-ketosteroids on fully acetylated paper. The lack of an impregnating agent and the very slight adsorption of these steroids on acetylated paper made exact determination possible. The standard deviations of cholestenone, progesterone and testosterone were determined, while several tests were made with prednisolone and hydrocortisone.

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