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QUANTITATIVE THIN-LAYER CHROMATOGRAPHY

ASSAY OF DRUG MIXTURES BY SCANNING OF REMISSION PEAKS

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SUMMARY

Several examples are given showing that scanning of the remission of thin-layer chromatograms may be successfully applied to the assay of drug mixtures not responsive to less complicated methods. Simultaneous assays of etofyllin and nicotinic acid, pentobarbital and cyclobarbital, as well as sodium oestrone sulphate and sodium equilin sulphate are discussed in detail. The importance of adequate spot preparation for scanning is pointed out. Figures on the accuracy and reproducibility of the procedures are given.

INTRODUCTION

In many cases therapeutically active substances in pharmaceutical products are mixed with other therapeutically active substances and/or excipients, which necessitates complicated methods for their isolation or separation. Although a sufficiently precise quantitative assay of such mixtures of pharmaceutical substances is sometimes possible, for instance by spectrophotometric measurements at various wavelengths or different pH values, sometimes the ratios of the amounts are so unfavourable, or the absorption curves so similar, that even this elegant method, which takes so little time, does not yield the desired results. Considering other methods, chromatographic separations require the least effort and among them thin-layer chromatography (TLC) has attained special significance. Besides semiquantitative evaluation by visual estimation of spot size, or quantitative evaluation after scraping off, elution and photometric measurement of the spots, optical measurements carried out directly on the thin-layer plate[1-8] have recently been developed.

EXPERIMENTAL AND RESULTS

In our laboratory, we have to deal with analytical problems concerning formulation, stability testing, and quality control. Some examples will demonstrate our experiences in solving these problems by means of direct evaluation of thin-layer plates, using a Zeiss spectrophotometer with the Camag attachment for thin-layer evaluation, the so-called Z-Scanner. We are able to solve most of our problems

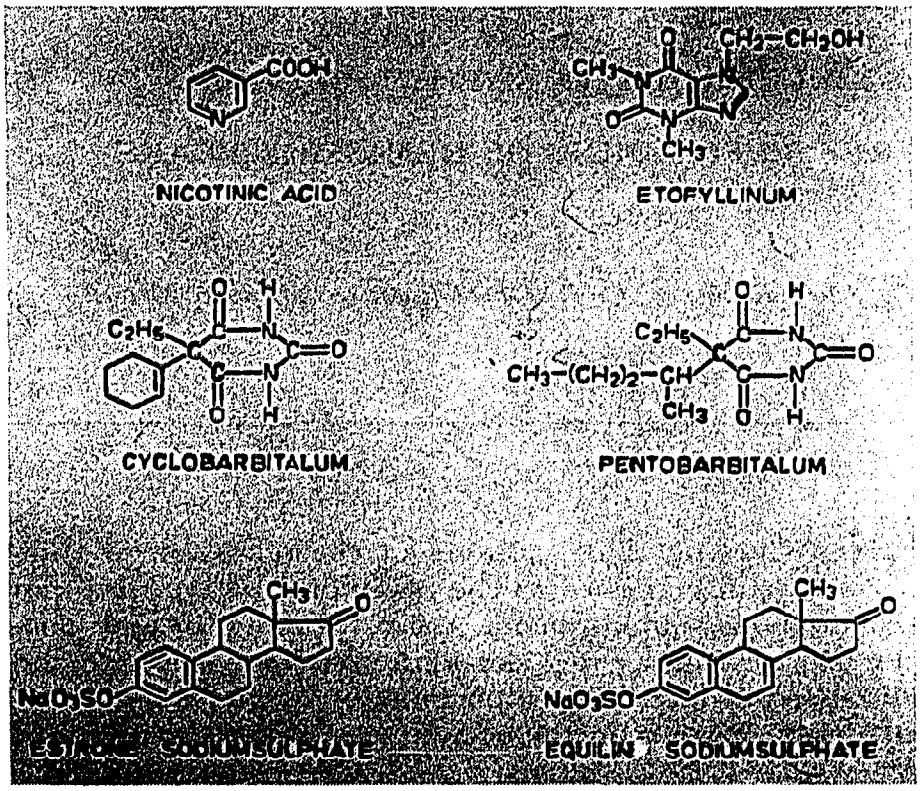

Fig. 1

concerning direct evaluation of thin layers by means of remission scanning with this apparatus and it is not necessary to revert to transmission measurements, fluorescence measurements, or fluorescence quenching measurements.

Fig. 1 shows the chemical formulas of the compounds whose quantitative assay was investigated. The first example is the simultaneous assay of nicotinic acid and etofyllin. It should be noted that etofyllin is a theophylline derivative frequently used in the G.F.R. The second example deals with the simultaneous assay of two barbiturates, *viz.* cyclobarbital and pentobarbital, used in relatively low concentrations in a night analgesic. The third example demonstrates the separation and quantitative assay of sodium oestrone sulphate and sodium equilin sulphate on thin-layer plates with silica gel impregnated with silver nitrate as a sorbent.

Assay of nicotinic acid and etofyllin

Prior to quantitative TLC, we used direct spectrophotometric determination, measurements of the two compounds being carried out at the two absorption maxima. While this method yields good results in pure mixtures of the two compounds, the measurements, according to our experience, were very inaccurate in coated tablets containing excipients. The solvent chloroform–ethanol–formic acid (8:1:1) permitted a good separation of the two substances. In this case, as for all other substances, the range where a straight line could be obtained for nicotinic acid by plotting the concentration against the integrated area of the logarithmically scanned remission was determined. In our measuring arrangement the Z-Scanner is connected to a Vitatron-recorder, which permits direct integration of the areas in logarithmic scanning.

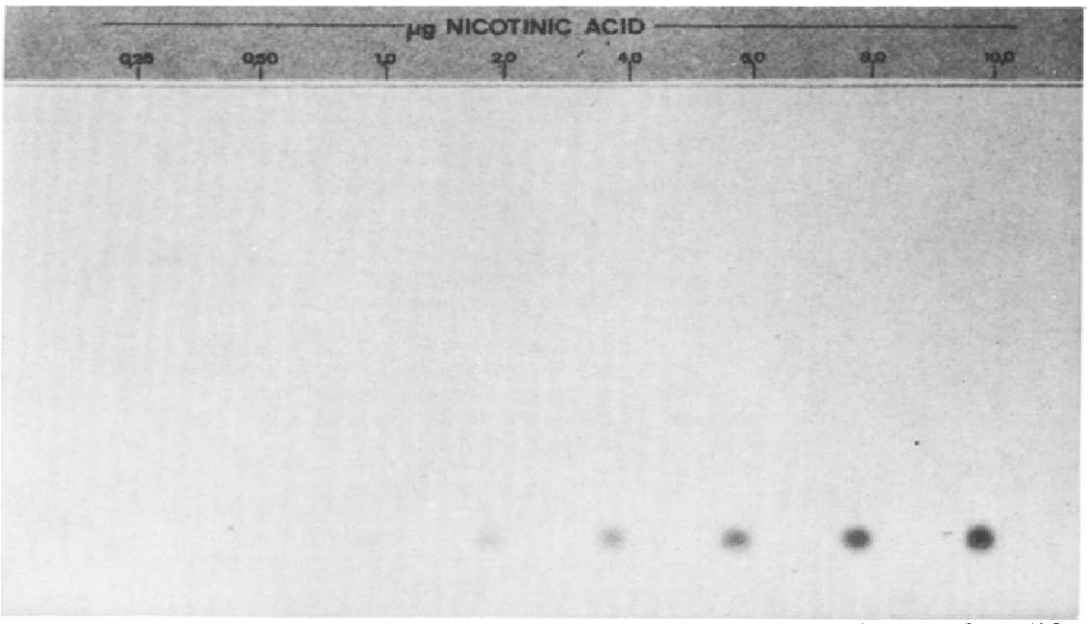

Fig. 2. TLC of nicotinic acid concentration series. Layer, Silica Gel F_{254} (Merck); solvent, chloroform–ethanol–formic acid (8:1:1); length of run, 10 cm; chamber saturation; time of run, *ca.* 45 min; pipetting volume, 2 μl.

Fig. 2 shows a concentration series of this type for nicotinic acid. The corresponding remission peaks are presented in Fig. 3.

Whenever possible, scanning was carried out at right angles to the direction of development, since this saves considerable time. However, in this case a well-run chromatogram is a prerequisite. As Fig. 2 shows, a straight line was obtained for nicotinic acid in the range 0.25 to 4 μg; this means that by using a standard, the evaluation can be directly related to it by means of the rule of three. The same applies to

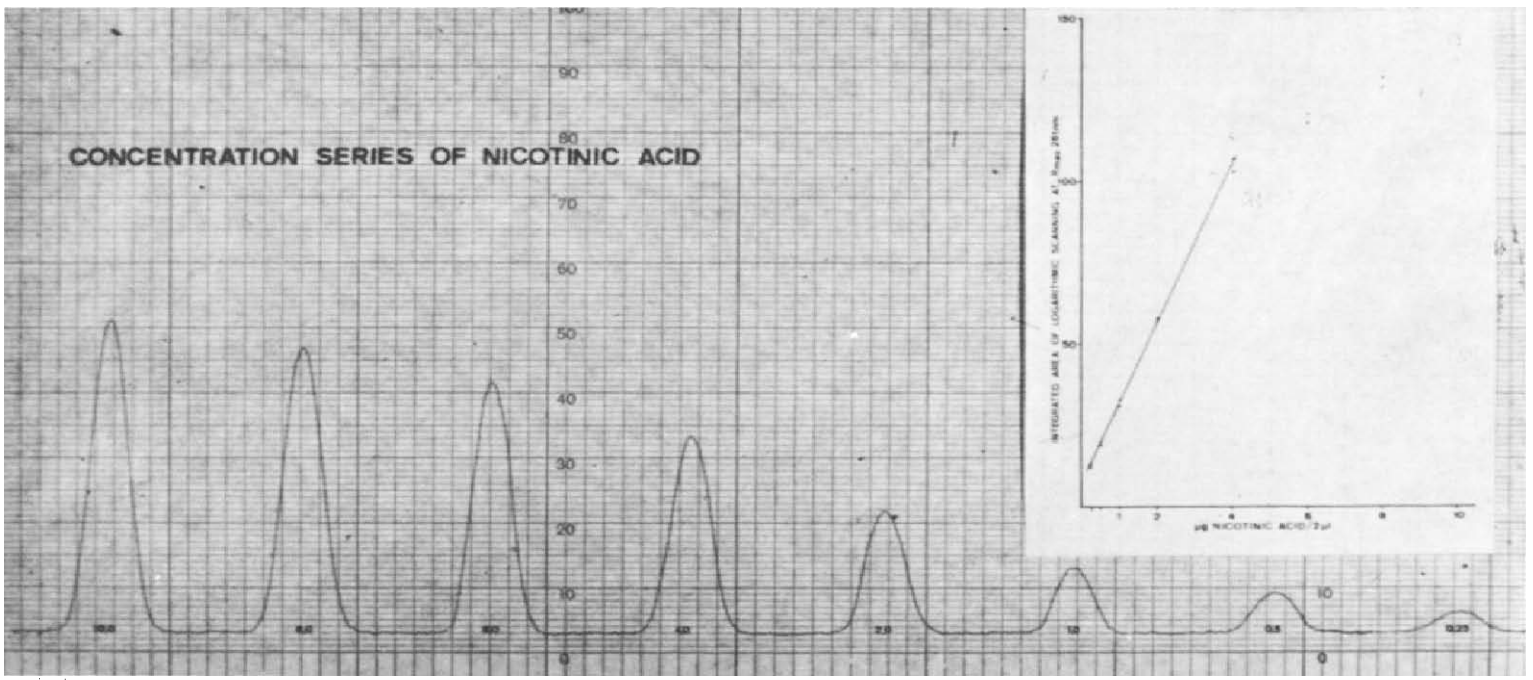

Fig. 3. Corresponding remission peaks of Fig. 2.

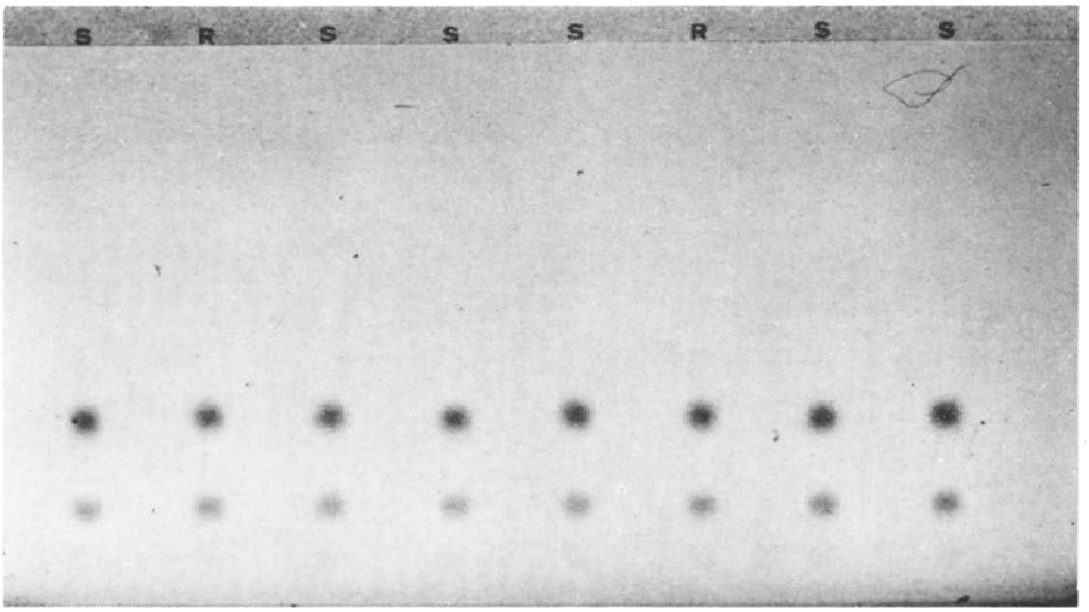

Fig. 4. TLC of nicotinic acid–ethofyllinum. Layer, Silica Gel F_{254} (Merck); solvent, chloroform–ethanol–formic acid (8:1:1); length of run, 10 cm; chamber saturation; time of run, *ca.* 45 min; pipetting volume, 2 μl; 0.690 g (3 coated tablets) per 100.0 ml methanol; sample(s), production lot 268; reference (R), 30 μg nicotinic acid and 60 μg etofyllinum.

etofyllin in the 0.5 to 8-μg range. Fig. 4 shows the separation of the two substances. In our product the weight ratio is two parts of etofyllin to one part of nicotinic acid. The upper spots represent etofyllin, the lower ones nicotinic acid. Nicotinic acid is scanned at 261.5 nm, etofyllin at 273 nm. Eight spots can be applied to a 20 × 20 cm plate; thus besides the two standards, six samples may be applied to one plate. Normally, we carry out three double assays, *i.e.* one per batch. In the case shown by this figure, the same batch was spotted six times, in order to permit a calculation of errors in relation to the standards. The values obtained are listed in Table I. The values for the coefficient of variation include the pipetting error; an amount of 2 μg each was spotted by means of so-called Barrolier pipettes.

Assay of cyclobarbital and pentobarbital

One of our products contains cyclobarbital in the form of the calcium salt and pentobarbital as the free acid. In this case the difference in solubility of these two compounds cannot be made use of for analytical purposes, since the product also contains aspirin. Due to its stronger acidity, aspirin binds the calcium ions of the cyclobarbital, so that solutions contain cyclobarbital as acid, having approximately the same solubility as pentobarbital. A simultaneous assay of the two barbiturates by titration with silver nitrate, followed by calcium titration with EDTA, is

TABLE I

RECOVERY AND CALCULATION OF ERRORS

	Nicotinic acid	*Etofyllin*
Theory	50.0 mg/tablet	100.0 mg/tablet
Found	50.4 mg/tablet	101.3 mg/tablet
Coefficient of variation	2.6	2.3

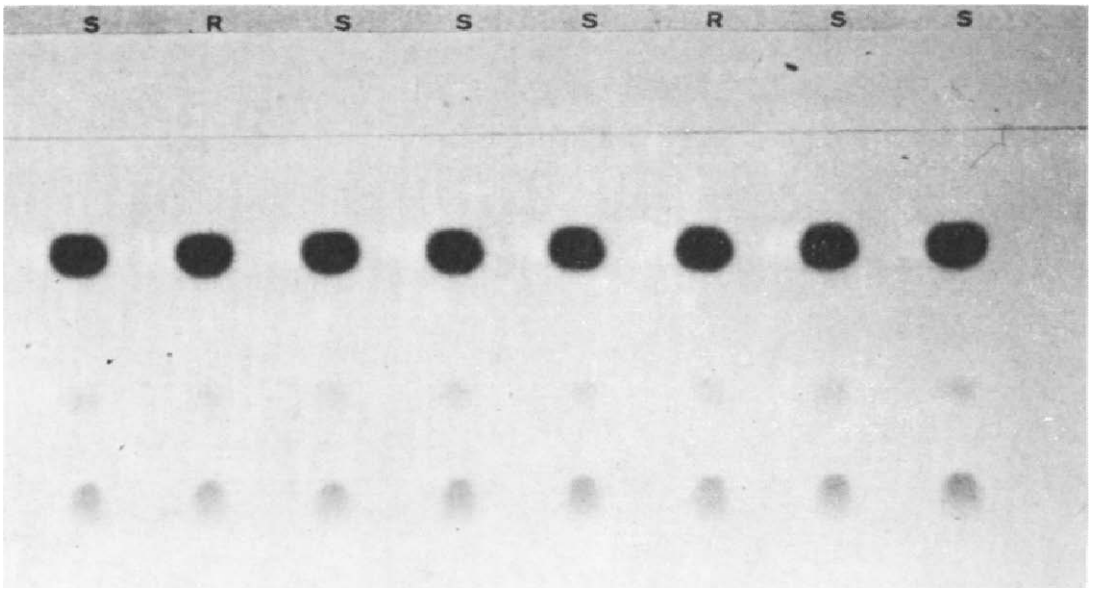

Fig. 5. TLC of cyclobarbitalum–pentobarbitalum. Layer, Silica Gel F_{254} (Merck); solvent, chloroform–isopropanol–ammonia (25%) (45:45:10; length of run, 8.5 cm; chamber saturation; time of run, *ca.* 45 min; pipetting volume, 5 μl; 4.9000 g (7 tablets) per 100.0 ml acetone; sample(s), production lot 412; reference(R), 8.75 μg pentobarbitalum and 1.75 μg cyclobarbitalum.

not particularly specific; the results obtained are also too inaccurate. Therefore, we again tried quantitative TLC. As shown by Fig. 5, the two substances can be separated in the solvent chloroform–isopropanol–25% ammonia (45:45:10). We found that with increased lengths of run, pentobarbital displays a tendency towards tailing and deformation of the spots. For this reason, we prefer a migration distance of only 8.5 cm. The quantity of barbiturates to be spotted is limited, since aspirin and phenac-

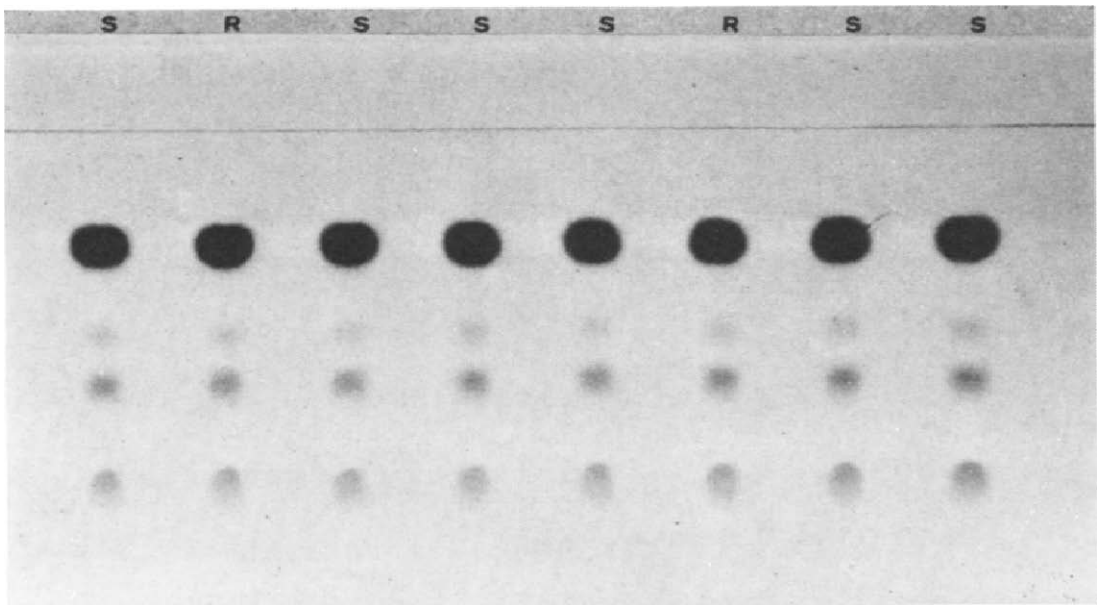

Fig. 6. TLC of cyclobarbitalum–pentobarbitalum. Layer, Silica Gel F_{254} (Merck); solvent, chloroform–isopropanol–ammonia(25%) (45:45:10); length of run, 8.5 cm; chamber saturation; time of run, *ca.* 45 min; pipetting volume, 5 μl; 4.9000 g (7 tablets) per 100.0 ml acetone; sample(s), production lot 412; reference(R), 8.75 μg pentobarbitalum and 1.75 μg cyclobarbitalum. 15-min treatment in a chamber saturated with ammonia.

etin are present at 4- to 8-fold concentrations in our product. Therefore we attempted to increase peak-area by other methods. As it is well known that at higher pH values barbiturates are subject to a bathochrome shift of the absorption maximum we therefore attempted to raise the intensity of the light absorption at relatively longer wavelengths by ammonia vapour. As can be seen in Fig. 6, this is possible by treating the sample for 10–15 min with ammonia vapours in a TLC chamber. This treatment yields spots of the two barbiturates in the 5- to 25-μg range, which can easily be evaluated. A straight-line relationship is again obtained by plotting the integrated area of the logarithmically scanned remission against the concentration. The increase in sensitivity of this method owing to the treatment with ammonia has the practical advantage that measurements can be carried out at 234 nm instead of 220 nm. This means that the slit width is reduced from 1.7 to 0.9 mm, which in turn means improved spectral resolution. In addition, the blank of the silica gel layer decreases at longer wavelengths. Using the method described above we obtained a coefficient of variation of 1.9 for cyclobarbital, and found a mean of 49.6 mg per tablet instead of 50.0 mg. In the case of pentobarbital the corresponding figures are: theory, 25.0 mg per tablet; found, 25.05 mg; coefficient of variation, 2.5.

It should be added that pentobarbital was the first substance we investigated for direct evaluation on thin-layer plates. Prior to deciding in favour of integration of the logarithmically scanned remission by means of the Vitatron recorder, we conducted comparative investigations on the possibilities of peak evaluation. We studied: (a) planimetry of linearly scanned peaks; (b) calculation of the linearly recorded peak areas according to the equation: base times height divided by 2; (c) cutting out and weighing the linearly recorded peak areas; (d) our method, *i.e.* integration of the logarithmically scanned peak areas. Although we found no differences regarding the coefficient of variation in the evaluation of eight such peaks, we were persuaded to work with method (d), due to the fact that in this method no time is consumed as compared to 15 to 25 min in the other methods mentioned.

In addition to the two barbiturates, well-separated spots of aspirin (below) and phenacetin (above) can be seen in Fig. 6. However, determination of the 75 μg of each of the two other substances is not possible, because the concentration–area relationship in this range is not linear and there is almost no slope. Quantitative assay of the phenacetin and aspirin could, however, be carried out on the plate if a smaller amount of these substances was spotted. On the other hand, analysis of these compounds by means of spectrophotometry is easier and time-saving.

Assay of sodium oestrone sulphate and sodium equilin sulphate

Both substances are found in the urine of pregnant mares. Together with other oestrogen derivatives, such as equilenin or oestradiol, which are present in this urine to a lesser extent, they have been used therapeutically for some time as so-called conjugated or esterified oestrogens. In addition to the so-called AOAC method, which was also included in the USP XVIII, a method for separation on Sephadex LH 30 has been reported[9] recently. Both methods are extremely complicated and require a particularly skilled analyst. In the AOAC method, the water-soluble conjugated oestrogens forming a mixture with other substances in the urine are separated by forming a complex with dicyclohexylamine acetate and are subsequently hydrolysed. Besides spectrophotometric assay of the free oestrogens obtained

in this manner, gas chromatography can also be employed. This, however, requires prior silylation or acetate formation, which again is tedious. Whether free oestrogens are present also in a raw material or in a pharmaceutical product owing to ester cleavage can only be decided by another, just as tedious procedure as for esterified compounds. In this case, however, the free oestrogens are partitioned by means of organic solvents. We are presently studying the problem as to whether the natural mixture of conjugated oestrogens can be substituted by a combination of synthetic sodium oestrone sulphate and sodium equilin sulphate. Since only two substances were involved, we expected to be able to simplify their analysis. According to studies carried out by DE VRIES[10], and later by IKAN AND CUDZINOVSKI[11] and DITULLIO *et al.*[12], sterol derivatives which differ only by an additional double bond, such as cholesterol and cholestanol, can be separated on silica gel if it is impregnated with silver nitrate. Since such a difference can also be found in the case of oestrone and equilin, we investigated whether the two substances also behave correspondingly. Primarily, our studies were to determine whether unhydrolysed compounds could be used directly, since this would be a further simplification of the method. A test for free oestrogens would not be necessary. The impregnation of regular silica gel layers on precoated plates is complicated, since, after this treatment with aqueous silver nitrate solution, the layers tend to come off, which, in turn, precludes proper scanning. However, we managed to impregnate Polygram foils (Macherey, Nagel & Co.) with a solution of 25 g silver nitrate in 75 g water, which provided a layer suitable for separation and direct quantitative assay. Fig. 7 shows this separation. Due to the low content of active substances in the pharmaceutical product, we had to spot relatively large volumes. Therefore we used the Linomat by Camag. As a consequence the spots are streak-shaped. Direct UV measurement of the two conjugated oestrogens is not possible, since UV rays alter the silver nitrate impregnated layer by reduction; we therefore tried staining the substances. Since any kind of

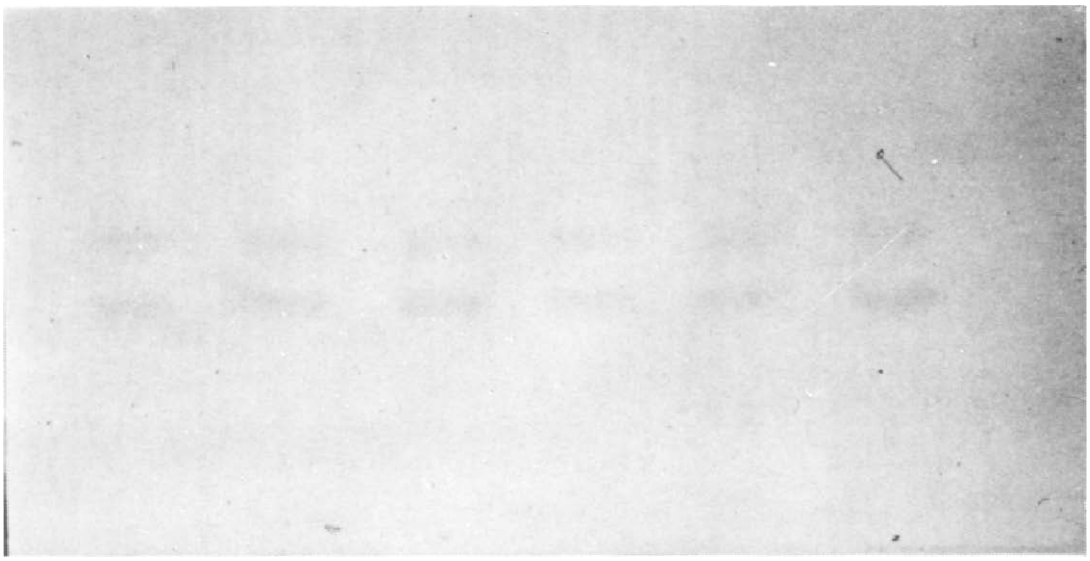

Fig. 7. TLC of sodium estrone sulphate–sodium equilin sulphate. Layer, silica gel (Polygram—Macherey, Nagel) precoated plastic sheet dipped in $AgNO_3$ solution (25%) at 30°; solvent butanol saturated with water; length of run, 10 cm; chamber saturation; time of run, *ca.* 120 min; pipetting volume: 50 μl sprayed on in bands by means of the "Linomat" (Camag/Muttenz, Switzerland); sample(s), 60 μg sodium equilin sulphate and 240 μg sodium estrone sulphate.

spraying requires a more or less non-uniform baseline, we exposed the plates to sulphuryl chloride vapours for 60 sec, followed by exposure to a steaming water-bath for 60 sec, following a suggestion of JONES *et al.*[13]. Subsequent drying of the plates for 10 min at 100° produces uniformly distributed sulphuric acid and hydrochloric acid on the plate, which results in the spots beginning to char. In our case, this resulted in colours permitting quantitative evaluation at 412 nm. Further charring again alters the baseline too much. Results obtained so far show that this staining method also has coefficients of variation between 2 and 5, the accuracy of the method being comparable to other scanning procedures. In this way we obtained a relatively simple method by which the content and the stability of substances in pharmaceutical products can be investigated.

It may be of interest that originally our studies included sodium equilenin sulphate. Due to a further double bond in ring B (Fig. 1), we expected an R_F value lower than sodium equilin sulphate. Surprisingly however, this was not the case, its R_F value being almost identical with that of sodium oestrone sulphate. This phenomenon is not restricted to conjugated oestrogens. It occurs with the free oestrogens as well; although differentiated by two double bonds, oestrone and equilenin yielded identical R_F values, with equilin being located lower than the two other substances. At present, there is no valid explanation for this behaviour.

Some data on the time consumed by these methods should be added. In the direct evaluation method, the expenditure of time is primarily due to the running time of the plates, which is 45–120 min, and to the time necessary for the preparation of the solutions, carried out by automatic shaking in suitable solvents for 60 min. During this time, the analyst is free to perform other duties. A total period of 3 to 4 h includes only 75 min of manual operations and allows the investigation of three batches, which means two standards and three double assays on one plate.

DISCUSSION

Summarising, it can be stated that direct evaluation of thin-layer plates is a useful method for the analysis of pharmaceutical products. The accuracy and reproducibility of the method can be considered good or at least satisfactory, while time consumption is not too high. It should be emphasised that proper preparation of the spots for measurement, for example by observance of a shorter running distance, exposure to ammonia and avoiding spraying by exposure to vapours, exerts a considerable influence on the sensitivity and accuracy of the measurement.

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