Partition chromatography of estrone, equilin and equilenin isolated from mares' urine*

Partition column chromatography has been extensively applied to the separation of both equine[1] and human[2–5] urines. HAENNI and associates[6], utilizing the sodium hydroxide impregnated Kieselguhr and benzene system of BANES and coworkers[1], separated estradiol-17β from estradiol-17α and the corresponding epimeric dihydroequilenins in a single chromatogram. HEARD and associates[7] applied the latter method to the separation of ketonic estrogens of mares' urine but required repetitive chromatography to separate estrone completely from its 7,8-unsaturated companion, equilin. This method was further modified by SAVARD AND BURDULIS[8] and provided satisfactory separation of estrone, equilin, equilenin and estradiol-17β in a single chromatogram, but left an intermixed portion of the estrone and equilin zones which amounted to approximately 13 to 18 % of the two compounds.

The object of this report is to describe a further modification of the method of SAVARD AND BURDULIS which provides a good separation of estrone and equilin in a single chromatogram by means of a slow gradient increase in the elution power of the mobile phase.

Methods and materials

Solvents. All solvents were reagent grade, freshly distilled; the ligroin boiling range was 70–110°.

Celite. No. 535 of Johns-Manville and Co. was treated according to BAULD[5].

Estrogen mixtures. These were extracted from the urines of pregnant mares after solvolysis and fractionation of the phenolic extract into ketonic and non-ketonic fractions. The ketonic phenolic fraction was chromatographed on silica gel and the estrogens eluted in a semi-purified form with ethyl acetate in benzene in a step-wise manner.

Apparatus. The chromatography tube is 50 cm long by 1.8 cm uniform inside diameter, fitted at the lower end with a finely ground stopcock. The mixing chamber consists of a 5 l cylindrical bottle, stirred magnetically and connected to the chromatogram tube by means of an inverted U-shaped glass tube (2 mm I.D.); the connection between the chromatographic column and the connecting tube is sealed by a rubber stopcock and the solvent is thereby siphoned from the mixing chamber to the upper part of the chromatographic column. The reservoir consists of a 500 ml separatory funnel; with the stopcock open, the funnel is allowed to feed into a glass column whose rate of flow is regulated by a finely ground stopcock held in position by a stopcock tension clip. The solvent in this column is held at a constant level by the position of the stem of the separatory funnel; this constant level of solvent permits a steady rate of the flow of solvent into the mixing chamber. Both the lower tips of the chromatographic tube and the glass column are of the same inside diameter (1 mm) (Fig. 1).

Under normal operating conditions the reservoir and the glass column are filled with benzene, the mixing chamber is charged with 2000 ml 29 % (v/v) benzene in ligroin.

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By adjusting the flow of benzene in the mixing chamber at one-fourth the outflow of mixed solvent to the column, the desired solvent gradient, which is calculated from the equation described by LAKSHMANAN AND LIEBERMAN[9], is achieved.

Packing of the column. 29 g of celite are placed in a mortar and covered with 180 ml of 29 % (v/v) benzene in ligroin; exactly 30 ml of *N* sodium hydroxide solution

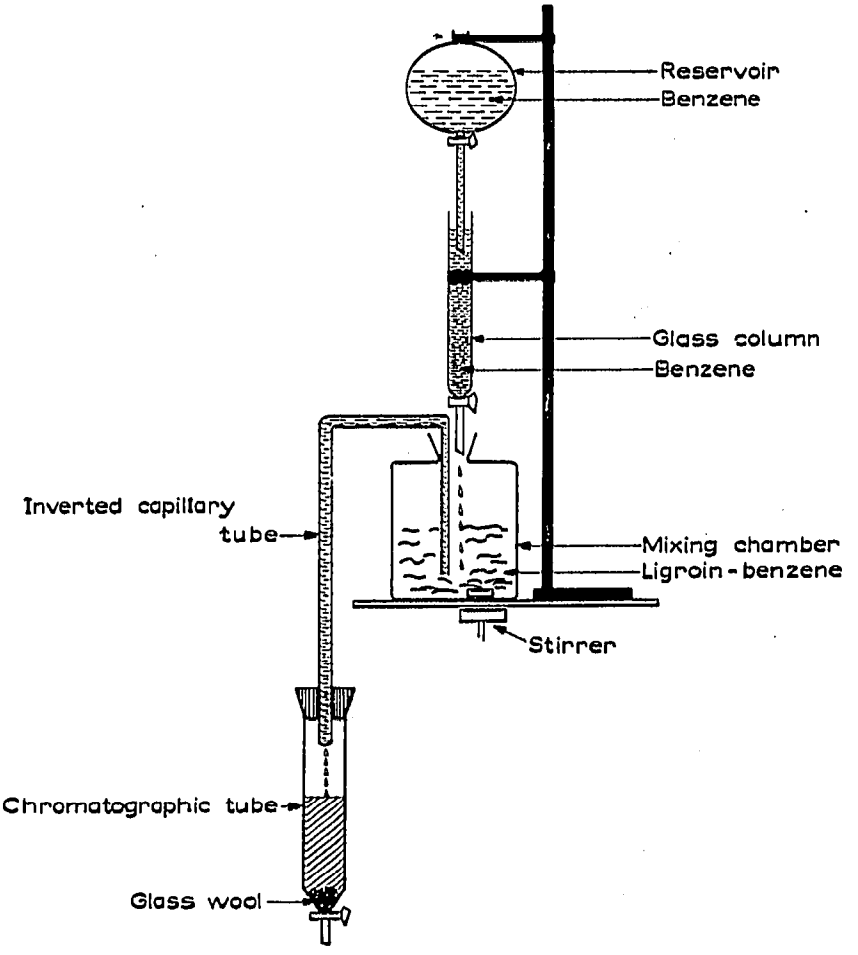

Fig. 1. Gradient elution chromatographic apparatus.

is distributed from a pipette over the Celite and the mixture is stirred for several minutes with a pestle until the celite appears uniformly wet. Meanwhile the chromatographic tube is one-quarter filled with solvent (29 % benzene in ligroin), a pad of glass-wool inserted and 1 g of celite, thoroughly impregnated with 0.5 water, is transferred to the column. The 29 g of sodium hydroxide impregnated celite is then transferred to the column and uniformly packed by means of a plunger as described by BAULD[10]. When complete the packed column is about 36 cm in height and the flow rate is 1.4 to 1.6 ml per minute.

Transfer of the steroid mixture to the column. The steroid mixture is dissolved in benzene and is mixed with 2 g of celite impregnated with 1 *N* sodium hydroxide solution (under ligroin–benzene). The solvents are allowed to evaporate and the steroid–celite mixture is transferred to the top of the prepared sodium hydroxide–celite column.

Running of the chromatogram. All chromatograms were run at 22–24° and 10 ml volumes of the eluate were collected. The steroids were quantitated in selected fractions by measuring their U.V. absorption in methanol.

Results and discussion

The initial composition of the mobile phase was selected after investigating the partition coefficients of estrone and equilin between 1 *N* NaOH solution and 50 and 30 % concentrations of benzene in various hydrocarbons (ligroin, heptane, methylcyclohexane). The level of 30 % benzene in saturated hydrocarbons affords approximately a two-fold difference in the partition coefficients of the two compounds in question. Accordingly the system 29–30 % benzene in ligroin was selected as the initial composition of the mobile phase.

It was observed that varying the volume of mobile phase in the mixing chamber and decreasing the concentration of benzene to 20 %, or both, did not improve the resolution of estrone and equilin zones, but actually increased considerably the threshold volume. Gradient variation in the concentration of benzene in the mobile phase, determined by the flow rates of solvent from reservoir to mixing chamber (R_1) and from mixing chamber to the column (R_2)[9] was found to give optimum resolution of estrone and equilin when the relationship of R_1/R_2 was $^1/_3$ or $^1/_4$ and R_2 was 1.4–1.6 ml/min. This provided on theoretical grounds a desirable concave shape to the solvent concentration curve, and in effect resulted in more symmetrically shaped elution curves for the principal estrogen zones than those obtained in the step-wise elutions described earlier[8].

Employing the conditions described in the methods section, up to 30 mg of a mixture of natural estrogens isolated from the urine of pregnant mares could be chromatographed on the sodium hydroxide impregnated celite partition column without any effect on the degree of separation or the symmetrical shape of the estrogens curves.

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