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The chromatographic separation of oestrone and equilin

Oestrone was first isolated from the urine of pregnant mares in 1931[1]. In the following year, two ring B-unsaturated 17-keto oestrogens, equilin[2] and equilenin[3], were isolated from the same source. There has always been difficulty in separating oestrone from equilin by chromatographic methods. Equilenin is easier to separate from equilin and it seems that any system separating oestrone from equilin will adequately separate equilenin from the other two. Information relating to the chromatographic systems reported to separate oestrone, equilin and equilenin is summarised in Tables I and II.

In the course of establishing a chemical assay for oestrone and equilin in the plasma of pregnant mares, the author developed a chromatographic system which gave an improved separation of oestrone and equilin. The details are as follows.

Whatman 3MM chromatography paper was cut into strips 3.5 cm wide and 50 cm long and washed before use by descending chromatography with absolute methanol for at least 24 h. Sample extracts or standard steroids were streaked over an area 3–5 cm from the end of the strip and "run-up" in the system ethyl acetate–methanol (2:1) to a pencil line drawn 6 cm down the strip.

Panglas 300 tanks (55 cm tall) were used for chromatography. They were always prepared freshly for each run or used only twice before the solvents were reshaken together and the tanks remade. After four or five runs the whole of the solvent system was renewed. Whatman 3MM paper stapled round glass rods was hung down one side of the chromatography tank into the bottom of the tank and down the opposite side into beakers placed on the bottom of the tank. 500 ml of redistilled G.P.R. ligroin 95–105° fraction, 450 ml of redistilled A.R. methanol and 50 ml of deionized water were shaken together in a separator and left to equilibrate. The lower phase was run down the 3MM paper into the beakers and the upper phase down the paper into the bottom of the tank. The papers with the samples on them were placed in the dry troughs at the top of the tank and allowed to hang into the body of the tank. The lid was sealed with sellotape and the system left to equilibrate for about 6 h in an insulated cabinet at 22 ± 2°. The chromatogram was developed with redistilled ligroin which had been equilibrated for at least 6 h over 90% methanol. The troughs were replenished as necessary. After about 34 h the papers were removed and hung up to air dry. The positions of the oestrone and equilin on the standard strip were detected by spraying the strip with a dilution of Folin and Ciocalteu's reagent (1:5) and exposing it to an atmosphere of ammonia[8]. The two steroids showed up as dark blue zones on a light blue background.

Table III shows the length of time for which the chromatograms were run, the mobilities of oestrone and equilin and the R_s of equilin to oestrone. It can be seen that the system not only very effectively separates oestrone from equilin but also separates them better than any of the systems previously described. The R_s of equilenin to oestrone is about 0.55.

The efficiency of separation was demonstrated in experiments using [6,7-^{3}H]-oestrone. About 95% of [6,7-^{3}H]oestrone applied to paper and chromatographed

(1971) 193–196

TABLE I

COLUMN CHROMATOGRAPHY SYSTEMS USED TO SEPARATE THE EQUINE KETONIC OESTROGENS

Stationary phase	*Elution phase*	*Fraction size*	*Elution speed*	*Fractions containing oestrone*	*Fractions containing equilin*	*Fractions containing equilenin*	*Reference*	*Notes*
31.5 g Celite saturated with 30 ml *N* NaOH	Ligroin–benzene (1:1) up to 75 fractions; then benzene	15 ml	1.5 ml/min	Variable but about 24–46	Variable but about 40–75	120–130	4 (adapted from 5)	Oestrone and equilin peaks separated by 25–30 fractions but overlapping amounted to 13–18% of the total.
600 g Celite saturated with 30 ml *N* NaOH	Skellysolve–benzene (1:1)	15 ml	30 ml/h	275–400	400–550	640–740	6	The slow flow rate was mandatory to separate oestrone from equilin.
200 g Celite impregnated with propylene glycol	Cyclohexane–benzene (1:1)	Not given	20 ml/h	Not given	Not given	Not given	6	Used to purify oestrone fraction from 600 g Celite column.

TABLE II

CHROMATOGRAPHIC MOBILITY ON PAPER OF THE EQUINE KETONIC OESTROGENS

Mobile phase	*Stationary phase*	*Oestrone*	*Equilin*	*Equilenin*	*Reference*	*Notes*
Benzene	Formamide	1.00 (0.78)[b]	0.95[a]	0.87[a]	7	
Benzene–chloroform (1:1)	Formamide	1.00 (0.87)[b]	0.97[a]	0.93[a]	7	
Cyclohexane–benzene (1:1)	Formamide	1.00 (0.35)[b]	0.88[a]	0.62[a]	7	
Dichlorobenzene	Formamide	1.00 (0.78)[b]	1.04[a]	0.93[a]	7	
Cyclohexane–benzene (2:1)	Methanol–water (3:3)	Not given	Not given	Not given	6	Used to purify equilenin fraction from 600 g Celite column (Table I); tanks 110 cm tall
40–60° fraction petroleum ether–benzene (2:1)	Methanol–water (4:1)	1.00 (0.45)[b]	0.95[a]	0.77[a]	7	Bush B_3 system
Cyclohexane–benzene (1:1)	Propylene glycol	Not given	Not given	Not given	6	Used to purify equilin fraction from 600 g Celite column (Table I)
Toluene	Propylene glycol	1.00 (0.38)[b]	0.91[a]	0.72[a]	7	

[a] R_s value
[b] R_F value.

TABLE III

CHARACTERISTICS OF THE CHROMATOGRAPHY SYSTEM DESCRIBED

Time for which the chromatogram ran (h)	*Mobility of oestrone (cm/h)*	*Mobility of equilin (cm/h)*	*R_s of equilin to oestrone*
36.0	0.94	0.73	0.78
37.0	1.10	0.86	0.78
33.0	1.07	0.86	0.80
35.0	1.22	0.93	0.76
35.0	1.22	0.97	0.79
32.5	1.13	0.86	0.76
32.5	0.98	0.77	0.79
33.0	1.05	0.84	0.80
32.75	1.22	0.94	0.77
32.5	1.10	0.86	0.78
32.25	1.25	0.98	0.78
Mean ± S.D.			
33.[illegible] .65	1.12 ± 0.105	0.873 ± 0.075	0.781 ± 0.01

could be recovered from the "oestrone" zone by elution with methanol and tritium could not be detected in significant quantities in the equilin fraction.

The system's major disadvantage was the time for which it had to run but in routine it proved possible to use the day during which one chromatogram was running to prepare a second batch of samples for chromatography.

Acting on a suggestion of Dr. R. V. SHORT (personal communication), attempts were made to improve the separation and thereby reduce running time by incorporating a volatile alkaline agent in the stationary phase. The aim was to parallel the use of sodium hydroxide-impregnated columns by SAVARD and others. However, when ammonia was used at various concentrations in the stationary phase no significant improvement resulted and difficulties in maintaining and remaking the tanks led to the discarding of ammonia based systems.

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