A NEW PAPER CHROMATOGRAPHIC SYSTEM FOR THE RESOLUTION OF 17-KETOSTEROIDS*

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INTRODUCTION

A new Zaffaroni type of paper partition chromatography was developed for the resolution of the 17-ketosteroids (17-KS) found in urinary extracts[1,2]. Two disadvantages frequently associated with the Zaffaroni systems are that development times of several days may be required to insure adequate resolution of isomeric steroids and then a prolonged drying period is necessary to remove the stationary phase in order to satisfactorily perform the identification tests. A system which employs dimethyl sulfoxide (DMS) and *n*-heptane avoids these difficulties and represents a considerable improvement in several other respects.

EXPERIMENTAL

The solvents and reagents used were of the highest purity available. The steroids were purchased from Mann Research Laboratories and U.S. Pharmacopeia Reference Standards Collection. Stock solutions of steroids 1 mg/ml were prepared in 90% aqueous ethanol and stored in the cold. Dimethyl sulfoxide, b.p. 189–191° and *n*-heptane, b.p. 98–99°, were obtained from Matheson, Coleman and Bell. These solvents were equilibrated against each other prior to use. *n*-Heptane was employed as the mobile phase and 50 v/v% DMS (1 vol. DMS plus 1 vol. methanol) as the stationary phase. The ambient temperature was maintained at 24 ± 1.5°.

Whatman paper No. 1 which had been maintained at a relative humidity of 56% at 25° was used. These strips were dipped into 50 v/v% DMS and then blotted with paper towels. They were then dried between fresh paper towels for 2 h prior to sample application. From 10 to 200 μg of steroids were applied with micropipets on a 10 × 5 mm zone, 11 cm from the end of a sheet. The drying time and zone size were controlled by directing a stream of nitrogen under the point of application. The strips were placed in glass chromatographic tanks 12 × 12 × 24 in. which had been

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pre-equilibrated with the solvents. Since the solvent-troughs contained 600 ml of mobile phase, a constant hydrostatic head could be maintained for extended periods.

Upon completion of the chromatographic run, the papers were inspected, and hung in a darkened fume-hood for an hour in order to remove most of the solvent. The sheets were then dried at 50° at 28 in. Hg overnight before being examined with ultraviolet light of 254 and 360 mμ wavelength, the Zimmerman test[3], the blue-tetrazolium test[3] and anisaldehyde–sulfuric acid–antimony trichloride reagent[4].

RESULTS AND DISCUSSION

The physical properties of dimethyl sulfoxide are consistent with the criteria established for a suitable stationary medium. It is a colorless neutral liquid which is transparent to U.V. light of 254 mμ wavelength. One can locate steroids containing a Δ^4-3-keto group by scanning the sheet immediately upon removal from the tank. The paper can be freed from the solvents by heating at 50° at 28 in. Hg for 12 h as compared to 18 h required for propylene glycol and several days for dimethyl formamide.

A system employing 50 v/v% DMS was found to be optimum as regards development time and ability to contain an adequate amount of sample. The use of higher concentrations increased the development time with no increase in loading capacity and resulted in a somewhat inferior resolution ability as compared to the standard system. The use of concentrations of DMS lower than 50 % possessed no particular advantage while their capacity for steroids was inferior to the standard technique.

The rate of migration of the mobile phase is a function of the type of paper, hydrostatic head, ambient temperature, the concentration of the stationary phase and the drying time for the chromatographic strip after impregnation. A detailed study of the last factor indicated that the most reproducible results were obtained when the sheets were dried for 2.0 ± 0.5 h.

A 5° increase in the ambient temperature caused a 10–30 % increase in the steroid mobility probably due to changes in the steroid partition coefficient. The relative mobilities for some typical 17-KS for both the *n*-heptane–DMS system and the ligroin–propylene glycol system (PML[5]) are assembled in Table I. The data, obtained at 24 ± 1.5°, are listed as relative mobilities with the reference steroids being deoxycorticosterone (DOC) and cortisone (CORT). Relative mobility is defined as the ratio of the distance travelled, per unit of time, by a given steroid to that of a reference steroid spotted on the same sheet.

The following program was used to resolve the following classes of 17-KS: compounds with an R_{DOC} of 1.0 or higher were developed for 2.5 h, while those with an R_{DOC} between 0.25 and 1.00 required a development time of 6–8 h, and steroids with an R_{DOC} of 0.20 or less 18–30 h, depending on the steroid*. Since DOC would have travelled off the sheet if used as reference for the last group CORT was used instead. The practice of employing a reference steroid was adopted not only to calculate relative

* These data were obtained when the conditions were so adjusted that *n*-heptane required 2.5 h to travel 45 cm and the migration rate for DOC was 5.0 ± 0.2 cm/h.

TABLE I

A COMPARISON OF THE RELATIVE MOBILITIES OF 17-KETOSTEROIDS OBTAINED BY TWO PAPER CHROMATOGRAPHIC SYSTEMS

Ambient temperature was 24° ± 1.5° for the DMS system.

	Compounds	Ligroin–propylene glycol[5]	Heptane–dimethyl sulfoxide	
		R_{AND}*	R_{DOC}**	R_{CORT}***
1.	3α-Hydroxy-5α-androstan-17-one (androsterone)	1.00	2.28	
2.	3β-Hydroxy-5β-androstan-17-one	1.00	2.25	
3.	4-Androstene-3,17-dione	1.70	2.05	
4.	3β-Hydroxy-5α-androstan-17-one (epiandrosterone)	0.70	1.85	
5.	3α-Hydroxy-5β-androst-9-en-17-one	0.70	1.80	
6.	3α-Hydroxy-5β-androstan-17-one	0.70	1.75	
7.	3β-Hydroxy-5-androsten-17-one (dehydroepiandrosterone)	0.70	1.35	
8.	5β-Androstane-3,11,17-trione	0.75	0.75	
9.	11β-Hydroxy-5α-androstane-3,17-dione	0.17	0.27	10.3
10.	3α-Hydroxy-5β-androstane-11,17-dione	0.26	0.20	6.7
11.	3α,11β-Dihydroxy-5α-androstan-17-one	0.10	0.12	3.5
12.	3β,11β-Dihydroxy-5β-androstan-17-one	0.048	0.09	2.25
13.	3β,11β-Dihydroxy-5α-androstan-17-one	—	0.075	2.15

* Mobility relative to androsterone as reference steroid.
** Mobility relative to deoxycorticosterone as reference steroid.
*** Mobility relative to cortisone as reference steroid.

mobilities, but also to establish whether the correct development time was used. Upon removal of a sheet from the chromatographic jar, it was inspected with a 254 mμ light source. If the reference steroid had travelled the correct distance, it would be removed for processing; however, if a longer development time was indicated, the run could be continued with no adverse effects.

The reproducibility of these data was established by a statistical analysis of 15 experiments in which the mobility of androsterone relative to DOC was compared. The mean R_{DOC} for androsterone was found to be 2.28 with a standard deviation of 0.15 or 7% of the mean. This is indicative of the order of reliability of the data. The major source of error is attributable to temperature fluctuations with its subsequent effect on the mobilities.

The ability of this system to separate steroids with almost identical mobilities cannot be explained solely in terms of small differences in distribution coefficients but rather as a manifestation of a mutual displacement effect[5]. This phenomenon occurs when two compounds with comparable mobilities migrate on the same sheet and one steroid will preferentially occupy all the sites thereby displacing the other and thus effect a physical separation. Illustrative of this is the experiment where a sample which contained 40 μg of 3α-hydroxy-5β-androst-9-ene, R_{DOC} = 1.80, 120 μg of 3α-hydroxy-5β-androstan-17-one, R_{DOC} = 1.75 and 40 μg of dehydroepiandrosterone, R_{DOC} = 1.35, were found to be distinctly separated from each other providing that the marker steroid, DOC, migrated 20 cm.

A comparison of the DMS system with the familiar ligroin–propylene glycol system (PML) is presented in Table I. The DMS system contained sets of 2 and 3

steroids with the same range of mobilities while the PML system contained 2 and 5 steroids, respectively*. With the DMS system the maximum development time was 30 h, while at least 7 days was required to achieve the same degree of resolution with the PML system. The latter system could handle a larger amount of steroid than the initial one. Up to 500 μg of crude urinary 17-KS have been processed successfully with the PML system; however, this amount of sample would overload the DMS system and cause considerable streaking.

These data supplemented with other unpublished results indicate that the DMS system is best suited for the resolution of steroids containing 1-3 oxygen substituents, present as hydroxyls or keto groups. The steroid esters, testosterone benzoate and androsterone benzoate, travelled with the solvent front and could not be resolved. Polar steroids such as corticosterone, cortisone, etc. were successfully resolved, but several days were required for development**.

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SUMMARY

A paper partition chromatographic system for the resolution of 17-ketosteroids is described, which employs *n*-heptane as the mobile phase and dimethyl sulfoxide as the stationary phase. This system has several advantages over the Zaffaroni-types in current use, namely a development time of 2.5–24 hours for 17-ketosteroids, it affords excellent resolution, and the solvents used do not interfere with the characterization tests.

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Editor's Note: According to the referees the *R* values of compound 3 in Table I are doubtful.

* It should be noted that all the steroids in both of these sets when present in mixtures, in reasonable concentrations, could be separated from each other by the DMS system apparently through the agency of the mutual displacement effect.

** At the time that this manuscript was being prepared for publication a report appeared indicating that organic acids could be resolved by a system similar to the one described here[6].