REFERENCES

(1) E. Gilmore, J. Weil, and C. A. Chidsey, *N. Engl. J. Med.*, **282**, 521 (1970).

(2) T. B. Gottleib, F. H. Katz, and C. A. Chidsey, *Circulation*, **45**, 571 (1972).

(3) G. E. Abraham and P. K. Grover, "Principles of Competitive Protein-Binding Analysis," W. D. O'Dell and W. H. Daughaday, Eds., Lippincott, Philadelphia, Pa., 1972, pp. 140–152.

(4) G. N. Trump, *Biochem. Biophys. Res. Commun.*, **54**, 544 (1973).

(5) T. J. Gilbertson, *J. Labeled Compd. Radiopharm.*, **12**, 463 (1976).

(6) M. E. Royer, H. Ko, J. A. Campbell, H. C. Murray, J. S. Evans, and D. G. Kaiser, *Steroids*, **23**, 713 (1974).

(7) D. Rodbard and J. E. Lewald, *Karolinska Symp. Res. Methods Reprod. Endocrinol., 2nd Symp.*, **1970**, 79.

(8) T. B. Gottleib, R. C. Thomas, and C. A. Chidsey, *Clin. Pharmacol. Ther.*, **13**, 436 (1972).

(9) R. C. Thomas, R. S. P. Hsi, H. Harpootlian, and R. W. Judy, *J. Pharm. Sci.*, **64**, 1360 (1975).

(10) R. C. Thomas and H. Harpootlian, *ibid.*, **64**, 1366 (1975).

(11) M. E. Royer, H. Ko, J. S. Evans, and K. T. Johnston, *Anal. Lett.*, **9**, 629 (1976).

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Evaluation and Optimal Combination of TLC Systems for Qualitative Identification I: Sulfonamides

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Abstract □ A mathematical criterion for the evaluation of chromatographic analysis procedures is given by the information content as derived from Shannon's equation. This information content yields a numerical value representative of the merits of each chromatographic separation and thus allows selection of the optimal systems. In most cases, however, one analysis is not sufficient to allow the qualitative identification of the sample. Therefore, several chromatographic systems are combined. Two approaches allow the desired combination; one either calculates the information content of several procedures as one mathematical value or classifies the systems according to mutual resemblance by numerical taxonomy techniques. From the resulting groups of dissimilar systems, one optimal system can be chosen per group according to the information content. The results obtained by these mathematical procedures are illustrated with a practical example: the selection and evaluation of systems for the TLC analysis of sulfonamides.

Keyphrases □ TLC—systems, evaluation and optimal combination for qualitative determination of sulfonamides □ Sulfonamides, various—TLC systems for qualitative determination, evaluation and optimal combination

TLC is one major method in pharmaceutical analysis for the identification of organic compounds, and there is an enormous literature on the subject. It is not always easy to select the best TLC systems from the many that have been published, and it is more difficult to select the optimal combination of two or more systems. The reasons for this difficulty are:

1. The systems (any combination of stationary phase and solvent) are developed by many different workers, who use slightly different development procedures, saturation conditions, *etc.*

2. Most investigators do not use objective value judgments but rather state that their separation procedures yield either "good" or "excellent" or "poor" results for a group of substances. Furthermore, while it is rather easy to characterize a separation of two substances, it is often more difficult to characterize a separation of 10 substances.

3. Even if the selection of the individually best systems is possible, it is often nearly impossible to obtain, on sight, the optimal combination, since the best combination of n systems does not necessarily contain the n individually best systems.

It is necessary to create some order out of this chaotic literature. One way to do this is to compile the literature available for restricted application domains (1). Another approach consists of a comparison under standardized conditions of reported systems (and, in the present case, of some new systems). Formal methods are then used for the evaluation and optimal combination of the TLC systems.

EXPERIMENTAL

Reagents and Chemicals—All solvents were reagent grade, and reference sulfonamides were used as 0.2% (w/v) solutions in acetone. Sulfanilamide was always used as an internal standard.

Adsorbent—Precoated TLC silica gel 60 F-245 plates[1] and precoated TLC aluminum oxide 60 F-254 plates[1] (type E) were used.

Detection was by UV light (254 nm).

Apparatus—The plates were developed in carefully controlled saturation conditions[2] and standardized at 40% relative humidity.

RESULTS

The separation systems proposed in the literature and a few others were investigated (Tables I and II). Table I contains those systems for which a preliminary screening with seven sulfonamides yielded unpromising results (bad streaking of the spots, all hR_f values near 0 or 100, *etc.*).

Table II lists the systems that passed the screening stage. The sul-

[1] Merck, Darmstadt, Germany.

[2] Vario-KS-Chamber, Camag, Muttenz, Switzerland.

Table I—Systems Investigated but Rejected after the Screening Test

Solvent	Stationary Phase	Reference
Chloroform–ethanol (80:15)	Aluminum oxide	2
Chloroform–2-propanol (80:15)	Aluminum oxide	2
Chloroform–1-butanol (80:15)	Aluminum oxide	2
Chloroform–propanol (80:15)	Aluminum oxide	2
Chloroform–1-pentanol (80:15)	Aluminum oxide	2
Chloroform–acetone (50:50)	Aluminum oxide	2
Chloroform–acetic acid (95:5)	Aluminum oxide	2
Chloroform–acetic acid (90:10)	Aluminum oxide	2
Ether–methanol (90:10)	Aluminum oxide	2
Ether–ethanol (90:10)	Aluminum oxide	2
Benzene–ethanol (80:20)	Aluminum oxide	2
Ethyl acetate saturated with water	Aluminum oxide	—
Chloroform–methanol (80:15)	Aluminum oxide	—
Methanol–1-pentanol–benzene (31:15:45)	Aluminum oxide	—
Acetone–methanol–25% ammonia (85:15:15)	Aluminum oxide	—
Acetone–methanol–25% ammonia (75:10:5)	Aluminum oxide	—
Acetone	Aluminum oxide	—
Ethyl acetate	Aluminum oxide	—
Ethanol	Aluminum oxide	—
Chloroform	Silica gel	—
Chloroform–methanol (95:5)	Silica gel	3, 4
Chloroform–methanol (90:10)	Silica gel	5
Chloroform–methanol (80:20)	Silica gel	6
Chloroform–methanol (70:30)	Silica gel	—
Chloroform–dioxane (95:5)	Silica gel	3
Chloroform–ethanol (80:10)	Silica gel	7
Chloroform–acetic acid (95:5)	Silica gel	—
Benzene–ethanol (90:10)	Silica gel	—
1-Butanol–water (90:9)	Silica gel	—
5% (v/v) Ammonia–1-butanol (50:50)	Silica gel	7
Methanol–water (96:8)	Silica gel	—
Methyl ethyl ketone–pyridine (75:5)	Silica gel	7, 8
Acetone–methanol–diethylamine (90:10:10)	Silica gel	7, 8
Diethylamine (absolute)–2-propanol–water (10:50:40)	Silica gel	9
Chloroform–methanol–butylamine (85:10:5)	Silica gel	3
Dioxane–ammonia–water (100:3:10)	Silica gel	10
Chloroform–methanol–acetic acid (94:5:1)	Silica gel	3
Cyclohexane–acetone–acetic acid (40:50:10)	Silica gel	7, 8
1-Butanol–acetic acid–water (30:30:30)	Silica gel	11
Methanol–1-pentanol–benzene (31:15:45)	Silica gel	12
Methanol–1-pentanol–benzene–water (31:15:45:7)	Silica gel	12
Acetone–butanol–water (20:50:30)	Silica gel	10
Acetone–benzene–water (65:30:5)	Silica gel	10
Benzene–1-butanol–pyridine (30:5:5)	Silica gel	13
1-Butanol–methanol–acetone–diethylamine (90:10:10:10)	Silica gel	14
Butyl acetate–1-butanol–acetone–10% ammonia (30:30:40:10)	Silica gel	15

fonamides in Table III were chromatographed with each system in Table II.

DISCUSSION

Evaluation of Individual Systems—To evaluate the systems objectively, it is necessary to assign a figure of merit to each of the 56 systems remaining after the screening test. This assignment is done by calculating I, the information content, using a procedure analogous to the one introduced by Massart (31). The R_f range is divided into m R_f groups of given class width (*e.g.*, 0.05 R_f unit); for each m group, there is a distinct probability, p_k, that the unknown sulfonamide will have an R_f value within the limits of this class. If there is an equal probability of occurrence for each sulfonamide in the set, the probability, p_k, of finding an R_f value from an R_f class containing r_k members of the n that comprise the complete set equals r_k/n.

The information content, expressed in bit, can then be described by Shannon's (32) equation:

$$I = -\sum_{i=1}^{m} \frac{r_k}{n} \log_2 \left(\frac{r_k}{n}\right) \qquad \text{(Eq. 1)}$$

For example, in System 1 (Table III), four R_f values are found in the first R_f class (0–0.04), one R_f value is found in the next class (0.05–0.09), *etc.* Therefore, the information content of the first R_f class equals $-(4/22) \log_2 (4/22) = 0.45$.

By addition of the information content calculated for each class, a global information content value is obtained, characteristic of the merit of the chromatographic system under investigation. The information content (expressed in bit) of each one of the 56 systems remaining after the screening test is given in Table III.

Selection of an Optimal Combination of Two or More Systems—For the selection of an optimal combination of two or more chromatographic systems, *i.e.*, a set of systems containing as much information as possible, two approaches have been proposed. A combination of the individually best systems is not necessarily appropriate, since often a number of those systems give the same information (correlated and, therefore, redundant information).

The first approach is to consider every possible combination of two or more systems and to calculate the quantity of information obtained. Such a procedure was introduced to calculate the information content of combinations of stationary phases in GLC (33) and to compute "the discriminating power" for individual systems and for each combination of two, three, or four systems in chromatographic and spectroscopic procedures (34).

The second approach is used here; it has the advantage of versatility. Comparisons of this method with the first approach (33, 34) were published elsewhere (35, 36). This second approach is based on the classification or clustering of chromatographic systems according to their resemblance, *i.e.*, according to similarities in their chromatographic behavior. Similar systems are grouped into one class; from each resulting group (with dissimilar chromatographic characteristics), the individually best system can be chosen according to an evaluation criterion such as the information content.

One such classification procedure is numerical taxonomy (NT). Its application was introduced recently to the choice of optimal sets of solvents in TLC (37). In classification by numerical taxonomy, an $n \times n$ similarity matrix is constructed, using, for instance, taxonomic distance (37, 38) or correlation coefficients to measure the resemblance between each pair of systems. The reduction of this matrix can be carried out by various grouping techniques, *e.g.*, by weighted (37) or unweighted (39) pair group methods using the arithmetic average.

In a first reduction step, the most similar systems, i and j, are selected,

Table II—Systems Selected after the Screening Test

Number	Solvent	Stationary Phase	Reference
1	Ether	Silica gel	16
2	1-Pentanol	Silica gel	—
3	1-Butanol	Silica gel	—
4	1-Hexanol	Silica gel	—
5	Chloroform–methanol (60:30)	Silica gel	—
6	Chloroform–methanol (100:30)	Silica gel	—
7	Chloroform–methanol (80:15)	Silica gel	7, 8
8	Chloroform–methanol (100:10)	Silica gel	16, 17
9	Chloroform–ethanol (80:15)	Silica gel	—
10	Chloroform–2-propanol (80:15)	Silica gel	—
11	Chloroform–1-butanol (80:15)	Silica gel	—
12	Chloroform–1-propanol (80:15)	Silica gel	—
13	Chloroform–1-pentanol (80:15)	Silica gel	—
14	Chloroform–acetone (50:50)	Silica gel	—
15	Chloroform–dioxane (80:20)	Silica gel	3
16	Chloroform–acetonitrile (50:50)	Silica gel	7
17	Chloroform–acetic acid (90:10)	Silica gel	—
18	Chloroform–hexanol (80:15)	Silica gel	—
19	Benzene–ethanol (80:20)	Silica gel	—
20	Benzene–ethanol (70:30)	Silica gel	—
21	Ether–methanol (90:10)	Silica gel	—
22	Ether–ethanol (90:10)	Silica gel	—
23	Ethyl acetate saturated with water	Silica gel	12
24	Ethyl acetate–methanol (90:10)	Silica gel	6, 18
25	1-Butanol saturated with water	Silica gel	10
26	1-Butanol–chloroform–diethylamine (70:70:10)	Silica gel	19
27	Chloroform–methanol–dimethylformamide (100:10:5)	Silica gel	20
28	1-Butanol–formamide–water (50:10:50) upper phase	Silica gel	10
29	Methyl isobutyl ketone–acetone–25% ammonia (25:100:25)	Silica gel	21
30	Chloroform–methanol–25% ammonia (90:15:2.4)	Silica gel	22
31	Chloroform–acetone–methanol–6 *N* ammonia (60:10:25:0.5)	Silica gel	23
32	25% Ammonia–1-methylpropanol–2-propanol–water (15:40:40:5)	Silica gel	9
33	25% Ammonia–1-methylpropanol–2-propanol (15:35:40)	Silica gel	9
34	Ethyl acetate–methanol–25% ammonia (85:15:15)	Silica gel	—
35	Ethyl acetate–methanol–25% ammonia (85:30:25)	Silica gel	—
36	Diethylamine–1-methylpropanol–2-propanol–water (15:40:40:5)	Silica gel	9
37	1-Butanol–chloroform–methanol–25% ammonia (40:15:15:15)	Silica gel	24
38	1-Butanol–chloroform–acetone–diethylamine (90:10:10:10)	Silica gel	24
39	Chloroform–methanol–acetic acid (90:5:5)	Silica gel	3
40	Chloroform–1-butanol–petroleum ether (30:30:30)	Silica gel	25
41	Chloroform–1-butanol–ether (10:10:10)	Silica gel	26
42	Chloroform–ethanol–pentane (35:30:25)	Silica gel	7
43	Chloroform–ethanol–heptane (10:10:10) plus 1.5% water	Silica gel	27
44	Chloroform–ethanol–heptane (10:10:10)	Silica gel	28, 23
45	Chloroform–1-butanol–acetone–formic acid (40:10:10:10)	Silica gel	15, 17
46	Cyclohexane–acetone–chloroform–ethyl acetate–ethanol (5:10:20:5:5)	Silica gel	5
A1	1-Butanol–water (1:1)	Aluminum oxide	29
A2	Chloroform–methanol (70:30)	Aluminum oxide	30
A3	Methanol–water (96:8)	Aluminum oxide	29
A4	1-Butanol saturated with water	Aluminum oxide	—
A5	Chloroform–acetone (30:70)	Aluminum oxide	—
A6	Acetone–25% ammonia (75:25)	Aluminum oxide	—
A7	Acetone–25% ammonia (80:15)	Aluminum oxide	—
A8	Ethyl acetate–methanol–25% ammonia (85:15:15)	Aluminum oxide	21
A9	Methanol–1-pentanol–benzene–water (31:15:45:7)	Aluminum oxide	—
A10	Acetone–methanol–25% ammonia (85:15:10)	Aluminum oxide	—

i.e., the systems showing the smallest taxonomic distance, Δ_{ij}, or the highest correlation coefficient, ρ_{ij}. These systems are considered to form one group, i'. The similarity between group i' and all other systems (*e.g.*, 1) is then calculated as follows (for instance for weighted pair grouping of distances):

$$\Delta_{i'1} = \Delta_{(i,j)1} = \tfrac{1}{2}(\Delta_{i1} + \Delta_{j1}) \qquad \text{(Eq. 2)}$$

A new $(n-1) \times (n-1)$ similarity matrix is constructed by reduction with one column and one row of the original matrix. This reduction is completed when all systems are linked to another system or group of systems in one nonoverlapping hierarchic system of groups and subgroups, eventually depicted in what is called a dendrogram.

Figure 1 represents the dendrogram resulting from a numerical taxonomic classification of the chromatographic systems in function of the taxonomic distance. Figure 2 shows the dendrogram in function of the correlation between the systems.

Systems 34–38 (Table III) are not taken into account for the numerical taxonomy classifications, because too many sulfonamides dissociate in two or more spots when developed with these solvent combinations.

Successive breaking of the links on the lowest three levels of the dendrogram yields, consecutively, two, three, and four groups of systems. In Fig. 1, the individually best systems (*i.e.*, with the highest information content) in each group are Systems 4 and A7, successively joined by Systems 29 and A9. A combination of these four systems allows identification of 20 out of 22 sulfonamides. System 29, however, was selected out of a group consisting of only one element. Therefore, the possibility exists that the chosen system indeed shows a peculiar chromatographic behavior but, nevertheless, has a poor separating capability.

To prevent this situation, Systems 29 and A9 are replaced by System A9 and one of the best systems out of the group formed by breaking off the link immediately following, or else by System A9 and a system that allows separation of both sulfonamides that could not be separated by the foregoing combination. Some of these systems are A4, 32, 33, and 45. A combination of Systems 4, A7, A9, and 45 (or 33, 32, or A4) yields an optimal separation pattern in which all sulfonamides under investigation can be identified by their R_f values.

The dendrogram in Fig. 2 yields a similar conclusion. The classification followed by selection of systems with the described procedure leads to a combination of System 4 with A7, successively followed by Systems 29 and A2 (two sulfonamides cannot be separated). Since System 29 is again selected out of a group containing only one element, the combination of 29 and A2 is replaced by A2 and the most informative system out of the group on the level immediately following, *i.e.*, System 45. The combi-

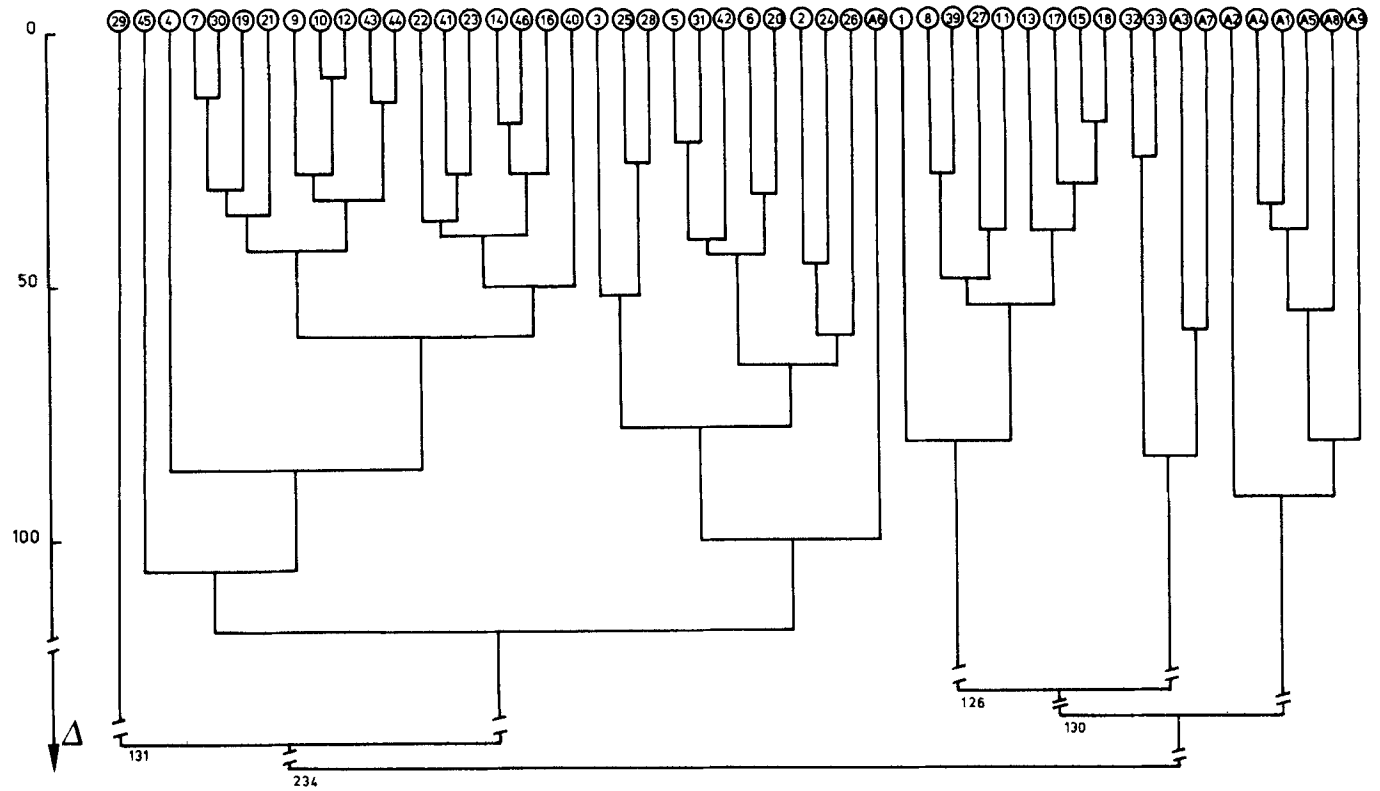

Figure 1—*Classification obtained by weighted pair arithmetic average linkage numerical taxonomy with the taxonomic distance, Δ, as the similarity parameter.*

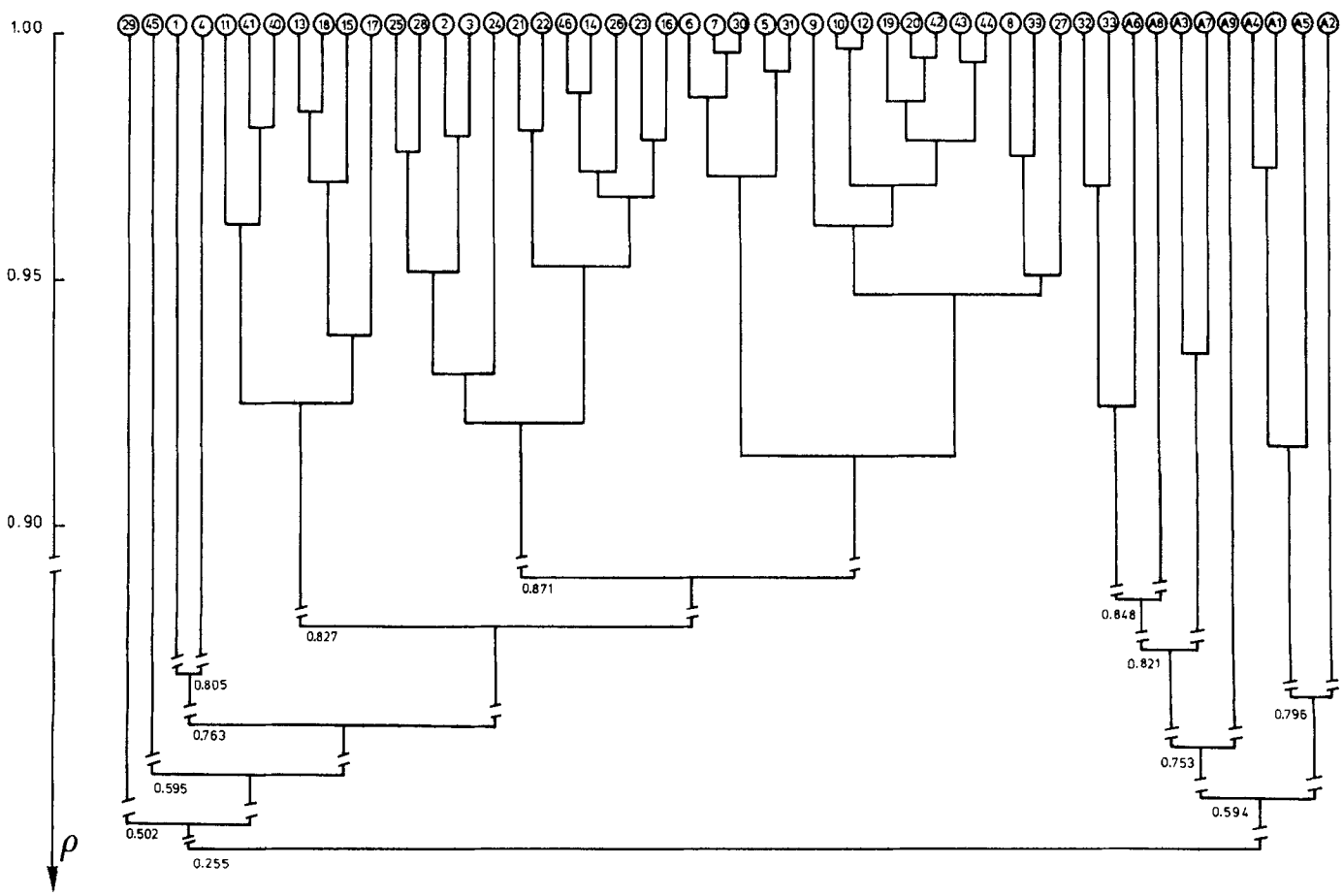

Figure 2—*Classification obtained by weighted pair arithmetic average linkage numerical taxonomy with the correlation coefficient, ρ, as the similarity parameter.*

Table III—R_f Values × 100 of the Sulfonamides in the Systems Selected after the Screening Test and Information Content of the Individual Systems (Expressed in Bit) (t = tailing)

Compound		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Sulfanilamide	1	29	75	85	61	67	54	35	18	35	31	23	30	18	55	16	59	9	16	37
Sulfacetamide	2	18	72	84	53	74	63	44	26	49	42	29	42	22	59	19	58	16	19	40
Sulfaguanidine	3	1	48	70	21	53	39	18	6	15	9	5	8	3	20	2	19	2	2	15
Sulfapyridine	4	24	69	79	47	79	73	57	38	61	53	38	52	29	63	24	60	36	25	50
Sulfadiazine	5	20[t]	63[t]	78[t]	24[t]	79	72	57	44	65	59	45	58	38	61	30	63	35	33	47
Sulfamerazine	6	26	66	80	33	79	73	60	42	66	66	51	63	40	66	34	67	37	36	53
Sulfathiazole	7	3	70	80	53	77	66	52	31	48	42	29	41	21	43	7	47	19	16	38
Isosulfamerazine	8	25[t]	68	79[t]	25[t]	79	71	57	43	66	67	54	67	42	67	34	70	38	39	56
Sulfamethazine	9	30	68	80	50	79	71	60	44	66	68	53	67	40	69	37	68	40	36	57
Sulfisomidine	10	8	26	52	11	78	72	55	34	46	34	20	32	12	37	12	25	13	10	32
Sulfaphenazole	11	36	95	97	89	79	73	60	43	66	69	63	72	54	68	42	80	36	47	59
Sulfisoxazole	12	35	84	93	74	76	66	55	32	62	61	48	58	38	70	31	72	27	29	53
Sulfamethizole	13	10	67	79	46	72	73	53	28	55	52	37	48	26	45	14	40	26	18	44
Sulfamethoxypyridazine	14	18	74	84	53	79	73	60	39	65	66	52	64	41	63	27	61	37	35	60
Sulfameter	15	18	73	84	55	79	72	60	39	66	66	38	66	46	67	33	70	37	37	58
Sulfadimethoxine	16	40	90	94	85	78	73	60	40	67	69	69	71	64	77	44	82	53	50	62
Phthalylsulfathiazole	17	1	0	18	2	29	16	5	2	3	3	2	2	1	2	0	0	4	0	0
Butylsulfanilureum	18	28	97	98	90	79	73	57	40	66	68	57	66	47	68	35	73	42	37	60
Tolbutamide	19	55	100	98	98	79	73	58	44	67	72	69	72	70	87	74	82	56	70	57
Chlorpropamide	20	26	91	98	84	78	73	58	43	57	71	71	73	70	73	57	80	56	56	61
Sulfamethoxazole	21	48	95	97	88	79	71	60	41	66	69	61	66	51	71	37	77	38	40	61
Succinylsulfathiazole	22	0	0	8	2	21	14	5	1	3	1	0	1	2	2	0	1	3	1	0
Information content		3.17	3.17	2.77	3.60	1.46	1.61	2.29	2.33	2.24	2.71	3.33	3.11	3.33	3.14	3.22	3.24	2.79	3.08	2.91

Compound		20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
Sulfanilamide	1	54	61	73	73	74	84	68	27	85	91	34	69	83	80	77	82	86	80	84
Sulfacetamide	2	60	54	67	66	71	85	75	38	85	25	44	72	38	32	9	39/79	39/86	41/84	86
Sulfaguanidine	3	31	19	15	16	50	49	15	11	69	73	18[t]	49	60	56	48	76	24/75	49	73/86
Sulfapyridine	4	68	58	70	72	72	81	75	45	81	92	57	74	52	46	76	80	45/80	82	85
Sulfadiazine	5	66	57	66	63	67	73	78	46	73	24	57	73	35	27	12	38/79	39/78	40	30/81
Sulfamerazine	6	70	57	66	68	70	80	85	48	81	90	57	75	38	31	77	81	51/82	44/86	85
Sulfathiazole	7	56	43	44	42	62	81	73	37	82	35	49	72	47	41	70	77	43/82	46/82	25/83
Isosulfamerazine	8	73	55	68	67	70	82	83	50	84	91	56	74	30	29	19	80	52	41/86	87
Sulfamethazine	9	72	57	74	73	74	80	81	49	81	91	57	74	43	35	75	81	58/82	45/87	87
Sulfisomidine	10	51	44	45	30	57	61	48	42	65	30	51	75	40	43	17	76	58	51	48/68
Sulfaphenazole	11	74	65	85	84	83	98	97	52	99	94	57	74	58	55	18	84	58/95	52	97
Sulfisoxazole	12	69	63	84	82	82	91	88	45	92	94	51	74	46	46	82/14	84	51/91	58/86	92
Sulfamethizole	13	60	45	63	53	65	79	75	38	78	87	50[tt]	69	44	42	69/13	43/78	45/80	48	30/82
Sulfamethoxypyridazine	14	72	55	65	66	57	83	85	49	84	94	57	75	43	39	73/23	80	57/87	89	88
Sulfameter	15	72	63	70	68	71	83	83	48	83	94	57	75	34	30	75/20	81	48/83	50/88	86
Sulfadimethoxine	16	73	62	85	83	84	95	97	53	94	95	57	75	44	47	78/24	85	57/91	53/95	95
Phthalylsulfathiazole	17	9	0	0	1	0	43	0	1	55	49	0	17	32	20	4/69	23	26/40, 86	37	8/84
Butylsulfanilureum	18	74	64	76	82	81	99	96	48	98	93	56	76	47	41	85	83	59/96	79/88	98
Tolbutamide	19	76	59	73	96	93	99	100	66	99	49	57	76	55	56	92	82	99	90	6
Chlorpropamide	20	75	60	76	84	80	98	100	66	99	92	57	75	60	60	79	82	96	79	97
Sulfamethoxazole	21	75	60	75	86	87	97	95	48	97	94	56	39	44	39	90	87	94	88	97
Succinylsulfathiazole	22	7	0	0	1	0	40	0	0	49	47	0	13	20	13	1	17	18	39	3
Information content		2.46	2.49	3.00	3.10	2.76	2.37	2.87	2.55	2.76	2.29	1.96	2.19	2.92	3.14	—	—	—	—	—

(continued)

Table III—(*Continued*)

Compound	39	40	41	42	43	44	45	46	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
Sulfanilamide	1 17	42	59	61	46	42	40	55	71	66	92	73	71	97	96	69	64	100
Sulfacetamide	2 26	47	64	67	50	50	62	61	2	4	40	6	2	58	37	3	12	9
Sulfaguanidine	3 6	16	25	41	29	26	22	24	38	48	74	52	31	92	90	48	49	92
Sulfapyridine	4 45	49	61	73	55	53	58	65	28	73	25	46	50	67	43	15	55	13
Sulfadiazine	5 45	52	64	74	55[t]	52	67	64	5	3	6	6	2	52	22	3	3	4
Sulfamerazine	6 48	58	69	82	61	60	72	66	4	7	11	9	5	57	18	5	17	7
Sulfathiazole	7 31	42	52	62	47	45	48	47	7	5	16	14	4	73	40	9	16	13
Isosulfamerazine	8 48	61[t]	70	84	65	63	74	68	5	4	10	8	10	55	18	4	14	4
Sulfamethazine	9 50	65	72	85	68	67	74	69	13	62	28	19	33	62	27	8	8	8
Sulfisomidine	10 38	26	38	63	45	45	27	45	10	20	51	12	7	69	49	8	25	18, 92
Sulfaphenazole	11 48	80	87	86	69	70	82	71	16	13	66	23	25	85	70	17	40	64, 97
Sulfisoxazole	12 38	64	80	78	58	59	72	71	4	4	52	8	2	76	48	12	17	30, 87
Sulfamethizole	13 39	47	59	71	47	50	64	54	2	2	10	4	1	69	36	8	7	14
Sulfamethoxypyridazine	14 45	60	69	82	68	64	70	67	10	50	12	20	3	61	26	10	38	12
Sulfameter	15 45	60	68	81	64	61	72	69	6	15	8	13	3	52	17	6	19	11
Sulfadimethoxine	16 52	83	85	85	75	73	83	72	10	34	35	18	4	77	52	18	36	33
Phthalylsulfathiazole	17 8	2	4	11	1	7	51	1	4	4	2	13	0	40	4	1	1	2
Butylsulfanilureum	18 45	84	87	87	73	70	80	69	5	7	17	12	4	70	27	13	21	11
Tolbutamide	19 50	94	94	89	79	77	44	80	5	4	28	11	2	90	35	22	48	16
Chlorpropamide	20 52	93	93	88	79	67	84	75	4	3	34	9	1	87	43	33	51	18
Sulfamethoxazole	21 44	81	86	86	69	7	80	68	3	2	13	6	3	74	33	8	14	14
Succinylsulfathiazole	22 8	2	5	12	0		42	2	0	1	2	1	0	44	1	0	2	0
Information content	2.57	3.24	3.39	2.68	2.95	3.15	2.92	2.88	2.31	2.73	3.35	2.59	1.85	3.26	3.41	2.60	3.39	—

nation of Systems 4, A7, A2, and 45 allows unambiguous identification of all 22 sulfonamides.

CONCLUSION

A practical conclusion is that, for the identification of sulfonamides by TLC, the following systems should be used in the order given: Systems 4, A7, A9 or A2, and 45 or 33, 32, or A4.

The best of all systems investigated is a very simple one (only one solvent). This practical conclusion confirms the experience of many practicing TLC or paper chromatography specialists that there is often no need for complex, multicomponent solvent systems and that good or, as in this case, even the best results are obtained with simple, easy-to-handle systems.

Our more general conclusion is that the use of formal methods for the evaluation and combination of TLC systems based on classification with numerical taxonomy, followed by selection of the individually best systems, leads to an optimal set of silica gel and aluminum oxide systems. This set allows the complete qualitative identification of commonly used sulfonamides.

REFERENCES

(1) G. H. Wagman and M. J. Weinstein, "Chromatography of Antibiotics," Elsevier, New York, N.Y., 1973.

(2) M. Sarsunova, V. Schwarz, E. Feketeova, and J. Protiva, *Pharmazie*, **21**, 219 (1966).

(3) M. Gajdos, *Cesk. Farm.*, **14**, 71 (1965).

(4) H. R. Klein and W. J. Mader, *J. Pharm. Sci.*, **60**, 448 (1971).

(5) K. C. Guven and O. Pekin, *Eczacilik Bul.*, **8**, 146 (1966); through *Chem. Abstr.*, **65**, 19928b (1966).

(6) E. G. C. Clarke and D. J. Humphreys, *J. Pharm. Pharmacol.*, **22**, 845 (1970).

(7) H. G. Gänshirt, in "Thin-Layer Chromatography," E. Stahl, Ed., Springer-Verlag, New York, N.Y., 1969, p. 544.

(8) W. Kamp, *Pharm. Weekbl.*, **99**, 1309 (1964).

(9) G. Ritschel-Beurlin, *Arzneim.-Forsch.*, **15**, 1247 (1965).

(10) R. D. Strickland, *J. Chromatogr.*, **24**, 455 (1966).

(11) K. C. Guven, S. Geçgil, and O. Pekin, *Eczacilik Bul.*, 8 158 (1966); through *Chem. Abstr.*, **65**, 18427d (1966).

(12) G. Wagner and J. Wandel, *Pharmazie*, **21**, 105 (1965).

(13) J. Pastor and R. Raimondi, *Trav. Soc. Pharm. Montpellier*, **23**, 220 (1963); through *Chem. Abstr.*, **62**, 11634 (1965).

(14) J. Reisch, H. Bornfleth, and J. Rheinbay, *Pharm. Ztg. (Frankfurt)*, **107**, 920 (1962).

(15) J. Zarnack and S. Pfeiffer, *Pharmazie*, **19**, 216 (1964).

(16) T. Bičan-Fister and V. Kajganovič, *J. Chromatogr.*, **11**, 492 (1963).

(17) J. L. Kiger and J. G. Kiger, *Ann. Pharm. Fr.*, **24**, 593 (1966).

(18) M. I. Walash and S. P. Agarwal, *J. Pharm. Sci.*, **61**, 277 (1972).

(19) J. Reisch, H. Bornfleth, and G. L. Tittle, *Pharm. Ztg. (Frankfurt)*, **109**, 74 (1964); through *Chem. Abstr.*, **63**, 16129g (1965).

(20) J. Pastor and R. Raimondi, *Bull. Soc. Pharm. Marseille*, **13**, 193 (1964); through *Chem. Abstr.*, **63**, 16133d (1965).

(21) M. T. Van der Venne and J. B. T'Siobbel, *J. Pharm. Belg.*, **18**, 557 (1963).

(22) T. Bičan-Fister and V. Kajganovič, *J. Chromatogr.*, **16**, 503 (1964).

(23) W. Kamp, *Pharm. Weekbl.*, **101**, 181 (1966).

(24) R. Neidlein, G. Klügel, and U. Lebert, *Pharm. Ztg.*, **20**, 651 (1965).

(25) N. Karpitschka, *Mikrochim. Ichnoanalyt. Acta*, **1**, 157 (1963).

(26) C. H. Pao, *Yao Hseuh Hseush Pao*, **13**, 67 (1966); through *Anal. Abstr.*, **14**, 3540 (1967).

(27) S. Klein and B. T. Kho, *J. Pharm. Sci.*, **51**, 966 (1962).

(28) E. G. Wollish, M. Schmall, and M. Hawrylyshyn, *Anal. Chem.*, **33**, 1138 (1961).

(29) J. Wandel, in "Thin-Layer Chromatography," E. Stahl, Ed., Springer-Verlag, New York, N.Y., 1969.

(30) L. R. Alexander and E. R. Stanley, *J. Assoc. Offic. Agr. Chem.*, **48**, 278 (1965).

(31) D. L. Massart, *J. Chromatogr.*, **79**, 157 (1973).

(32) C. E. Shannon, "A Mathematical Theory of Communication," *Bell Syst. Tech. J.* (1948).

(33) P. F. Dupuis and A. Dijkstra, *Anal. Chem.*, **47**, 379 (1975).

(34) A. C. Moffat and K. W. Smalldon, *J. Chromatogr.*, **90**, 9 (1974).

(35) A. Eskes, P. F. Dupuis, A. Dijkstra, H. De Clercq, and D. L. Massart, *Anal. Chem.*, **47**, 2168 (1975).
(36) H. De Clercq and D. L. Massart, *J. Chromatogr.*, **115**, 1 (1975).
(37) D. L. Massart and H. De Clercq, *Anal. Chem.*, **46**, 1988 (1974).
(38) P. H. A. Sneath and R. R. Sokal, "Numerical Taxonomy," Freeman, San Francisco, Calif., 1973, p. 124.
(39) H. De Clercq, D. Van Oudheusden, and D. L. Massart, *Analysis*, **3**, 527 (1975).

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Comparative Pharmacokinetics of Coumarin Anticoagulants XXIX: Elimination Kinetics and Anticoagulant Activity of (*S*)-(−)-Warfarin in Rats before and after Chronic Administration

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Abstract □ The kinetics of elimination and the anticoagulant effect of (*S*)-(−)-warfarin were determined in adult male rats before and after daily drug administration for 13 days. There was a small but statistically significant ($p < 0.05$) decrease in the body clearance of (*S*)-(−)-warfarin (from 4.84 to 4.37 ml/hr/kg) and an increase in the serum free fraction of racemic warfarin (added to serum *in vitro*) from 0.00850 to 0.0107 ($p < 0.05$). The concentration of (*S*)-(−)-warfarin in serum at which the synthesis rate of prothrombin complex activity is one-half of the prewarfarin rate increased from 0.532 to 0.655 μg/ml on the average ($p < 0.05$).

Keyphrases □ Warfarin—elimination kinetics and anticoagulant activity, effect of chronic administration, rats □ Elimination kinetics—warfarin, effect of chronic administration, rats □ Anticoagulants—warfarin, elimination kinetics and activity, effect of chronic administration, rats □ Coumarins—warfarin, elimination kinetics and anticoagulant activity, effect of chronic administration, rats

The coumarin anticoagulants act by inhibiting the synthesis of the vitamin K-dependent clotting factors II (prothrombin), VII, IX, and X. This inhibitory effect is accompanied by the formation of so-called abnormal prothrombin in humans (1–4), oxen and cows (5–8), and rats (9–12). In humans treated with a coumarin anticoagulant, abnormal prothrombin can be detected within 8–12 hr after drug administration and becomes the predominant form of prothrombin in plasma after 24–84 hr (3). The earlier investigations suggested that abnormal prothrombin has no coagulant activity; more recently, it has become apparent that there are several abnormal prothrombins and that some do have activity, but considerably less than that of normal prothrombin (4, 8). Apparently, abnormal prothrombin is a precursor of normal prothrombin and accumulates during treatment with coumarin anticoagulants, because these vitamin K antagonists interfere with the conversion of the precursor to its fully biologically active form (9, 11, 12).

The clinical implications of the accumulation of abnormal forms of prothrombin during chronic treatment with dicumarol or warfarin are not known. In view of the potential hazards of conducting such studies in humans, an investigation was carried out in rats to determine the relationship between the anticoagulant effect and the warfarin concentration in plasma before and after chronic drug administration. While the results may differ quantitatively from those in humans, it is considered likely that they will reflect in principle the events that may be encountered clinically.

EXPERIMENTAL

This investigation was carried out in five phases: (*a*) screening of rats for serum protein binding of warfarin, (*b*) administration of a single large dose of warfarin to rats whose serum free fraction of warfarin varied widely and determination of the time courses of drug concentration and anticoagulant activity in plasma, (*c*) daily administration of a maintenance dose of warfarin to these rats for 13 days, (*d*) administration of a second large dose of warfarin and determination of the time courses of drug concentration and anticoagulant activity in plasma, and (*e*) determination of serum protein binding of warfarin.

A 3-ml blood sample was taken from the tail artery of 26 adult male Sprague–Dawley rats, and the serum was separated. The serum was spiked with racemic ^{14}C-warfarin, about 1 μg/ml, and the free fraction was determined by equilibrium dialysis (13).

Based on the results of the screening study, 12 rats with widely differing serum free fraction values for warfarin were selected. Their body weights ranged from 350 to 440 g during all phases of the investigation. They received a 0.6-mg/kg iv injection of ^{3}H-(*S*)-(−)-warfarin (specific activity, 1.43 mCi/mg).

Blood samples (0.45 ml) were taken serially from the tail artery until prothrombin complex activity had returned to between 60 and 80% of the prewarfarin level. Plasma warfarin concentrations were determined by scintillation counting after extraction and TLC using a slight modification of a previously described method (14). To 0.2-ml samples of plasma was added 5 μl of unlabeled (*S*)-(−)-warfarin, 1 mg/ml, in acetone solution. The samples were then acidified and extracted with 2.5 ml of ethylene dichloride from which 2 ml was evaporated under nitrogen for chromatography (14). Recovery of ^{3}H-(*S*)-(−)-warfarin from spiked samples was 88.3 ± 2.4% (mean ± *SD*, $n = 16$) in the 0.013–6.33-μg/ml concentration range and was independent of concentration. Determinations of prothrombin complex activity and pharmacokinetic calculations were carried out as previously described (14).

After completion of the single-dose warfarin study, the rats received daily injections of ^{3}H-(*S*)-(−)-warfarin, 83–98 μg/kg ip, for 13 days to maintain prothrombin complex activity synthesis rate (R_{syn}) at about 30% of normal.

Two days after the last maintenance dose, the rats received another