Thin-layer chromatography of drugs in medicated feeds

Of the more than 40 million tons of feed manufactured annually in the United States, a very large percentage contains one or more drugs. Accurate, specific, and rapid methods of determining these drugs in feeds are needed for regulatory work. The Feed Additive Compendium[1], an authoritative manual for the feed industry, contains a compilation of various qualitative tests and assay procedures from the Manual of Microscopic Analysis of Feeding Stuffs[2], Official Methods of Analysis[3], and other sources. However, the majority of the tests described are generally nonspecific and not applicable to multicomponent preparations. In addition, many of the quantitative assay procedures for medicated feeds, *e.g.*, from Official Methods of Analysis[4], are themselves quite nonspecific.

R_F values obtained by thin-layer chromatography (TLC) have been reported for some of these drugs, namely, reserpine[5], propionic acid[6,7], griseofulvin[8,9], tetracyclines[10], penicillins[10,11], erythromycin[12], ronnel[13], and diethylstilbestrol[14].

The purpose of the work reported here was to extend the versatility, rapidity, and relative specificity of TLC to some of the other drugs commonly used in medicated feeds. Many of these drugs are commonly used in combinations such as ethopabate with amprolium. A number of these common multicomponent drug mixtures were separated and identified by TLC. To facilitate their identification the drugs were placed in categories according to similarity of structure and physical properties.

A procedure is described for the identification of 18 ingredients and 7 mixtures commonly used in medicated feeds. Various indicators have been used and lead–manganese–activated calcium silicate was incorporated in the preparation of the adsorbent to increase visibility of the spots and sensitivity of the procedure.

It should be noted that the data in this paper were obtained by working with standards of acceptable purity. Extraction and purification procedures for the drugs incorporated in the feeds are being developed and will be reported later.

These procedures will yield extracts of the feeds suitable for chromatography.

Experimental

The phenylarsonic acids were dissolved in 2 ml of 0.5 *N* sodium hydroxide and 98 ml of water. All the other reference standards were dissolved in dimethyl sulfoxide. N,N-Dimethylformamide can also be utilized as a solvent for the nitrophenyl compounds. All standards were stored in "Low-Actinic" glass-stoppered flasks to inhibit decomposition.

Preparation of plates

The adsorbent suspensions were applied to the plates with a Desaga applicator to a uniform thickness of 250μ. The plates were kept at room temperature for at least 30 min, then were activated at 100–110° for 30 min.

Alumina G. The suspension for five 20 × 20 cm plates or fourteen 10 × 15 cm plates was prepared by mixing 30 g of Alumina G (Camag DS 5) and 55 ml of water for 45 sec.

Alumina GF. A fluorescent indicator* (lead–manganese–activated calcium

* Distillation Products, Inc., Rochester, N.Y.

silicate) was added to Alumina G in a concentration of 300 mg/30 g (1 %) and a suspension was prepared as above.

Silica Gel G. A suspension of 30 g of Silica Gel G (E. Merck, Darmstadt) and 60 ml of water was prepared as for Alumina G.

Silica Gel GF. The fluorescent indicator (see Alumina GF) was added at a concentration of 1 % and the suspension was prepared as for Silica Gel G.

Polyamide G. A suspension was prepared by mixing 4.5 g of polyamide (Woelm TLC) and 500 mg of 100-mesh precipitated calcium sulfate ($CaSO_4 \cdot 2H_2O$) with 45 ml of methanol for 1 min.

Polyamide GF. 50 mg of the fluorescent indicator was added and a suspension was prepared as for Polyamide G.

Silica Gel GF on flexible plastic sheets. Eastman Chromagram Sheets, Type K 301 R, obtained from Distillation Products, Inc., Rochester, N.Y., were activated by heating for 30 min in a forced-air oven at 100° and were stored in a desiccator over silica gel.

General conditions

Table I shows the adsorbent and the developing solvent systems used in this study.

For reproducibility of the R_F values, the amount of drug applied to the plate should be in the range of 0.5–1.5 μg. The origin should be at least 2 cm from the bottom edge of the plate. It was also necessary to have the chromatographic chamber completely saturated with the solvent. To accomplish this the walls were lined with filter paper which dipped into the solvent. After 15–30 min the chamber became saturated with solvent vapor.

For solvent systems in which a volatile acid or base was used, the plate was

TABLE I

SOLVENT AND ADSORBENT SYSTEMS FOR THIN-LAYER CHROMATOGRAPHY OF DRUGS USED IN MEDICATED FEEDS

System No.	*Mobile solvent*	*Proportions*	*Min/ 10 cm*	*Adsorbent*
1	*n*-Butanol–water–acetic acid*	80:20:2.5	90	Polyamide G or GF
2	Acetone–acetic acid	100:2	15	Alumina G or GF
3	Ethanol–ammonium hydroxide	80:20	80	Alumina G or GF
4	Diethyl ether–dimethyl sulfoxide–*n*-butanol–acetic acid	98:1:1:0.2	15	Silica Gel G or GF
5a	Hexane–acetone–*n*-butanol	21:2:2	25	Silica Gel GF (flexible plastic sheet)
5b	Hexane–acetone–*n*-butanol	21:2:2	25	Silica Gel GF
6	Diethyl ether–ethanol–acetic acid	96:3:1	25	Polyamide GF
7	Acetone–*n*-butanol–acetic acid	78:20:2	20	Polyamide G or GF
8	Diethyl ether–*n*-butanol–acetic acid	73:25:2	20	Polyamide G or GF
9	*n*-Butanol–water–acetic acid	80:20:5	90	Silica Gel G or GF
10	Ethyl acetate–acetic acid	75:25	60	Silica Gel G or GF
11	Pyridine–benzene	50:50	45	Silica Gel G or GF
12	*n*-Butanol–acetic acid	75:25	60	Silica Gel G or GF

* The mixture was shaken in a separatory funnel and the lower layer was discarded.

heated in a forced-air oven for 30 min to remove all traces of the acid or base before using the detection system.

*Detection systems**

D1. A 5 % w/v solution of potassium hydroxide in methanol was prepared 2 h before use. If the spots were not visible after spraying with this reagent, the plate was viewed under U.V. light.

D2. A saturated (*ca.* 0.2 %) solution of barium diphenylamine-4-sulfonate** in methanol was prepared by first dissolving the salt in a few ml of N,N-dimethylformamide and then diluting to volume with the methanol[15]. The plates were heated at 110° for 10 min after spraying, and then were viewed under U.V. light.

D3. The plates were subjected to U.V. light.

D4. A solution was prepared by dissolving 20 mg of 4-methylumbelliferone[15] in 35 ml of ethanol, and then diluting to 100 ml with water. After the plates were sprayed, spots were seen under U.V. light. Exposure of the plates to ammonia vapor may aid in visualization of the spots.

D5. A solution of 0.2 % β-naphthoquinone-4-sulfonic acid (sodium salt)[15] in 5 % sodium carbonate was prepared 10–18 min before use. The plates were viewed under U.V. light after spraying.

D6. A 1.0 % solution of *p*-dimethylaminobenzaldehyde was prepared in ethanol which contained 1 % hydrochloric acid.

D7. A saturated (*ca.* 22 %) solution of antimony trichloride[15] in dried chloroform was prepared. This spray must be freshly prepared. The plates were sprayed, then heated at 110° for 15 min. If the spots were not visible, the plates were viewed under U.V. light.

D8. A 2 % solution of vanillin[15] in isopropanol was prepared. The plates were heated at 110° for 10 min after spraying.

Results and discussion

Table II lists the R_F values for the drugs in their respective adsorbent and solvent systems. The detection systems for the various classes of compounds are shown in Table III. When the phenylarsonic acids were chromatographed on polyamide (Table II), it was necessary to prepare the adsorbent with 10 % gypsum binder to increase the separation between carbarsone and 4-nitrophenylarsonic acid. The methanolic potassium hydroxide (Table III) was found to be a suitable secondary chromogenic agent, but did not have the sensitivity ($\leqq$ 0.25 μg) of 4-methylumbelliferone (D4). *p*-Dimethylaminobenzaldehyde (D6) was also a suitable detection agent for arsanilic acid. 4-Nitrophenylarsonic acid and 3-nitro-4-hydroxyphenylarsonic acid can be detected by barium diphenylamine-4-sulfonate (D2). 3-Nitro-4-hydroxyphenylarsonic acid can also be detected by U.V. light.

On the thin-layer chromatograms of nitrofuraldehydes (Tables II and III), furazolidone fluoresced and nitrofurazone quenched the fluorescence of U.V. light before any detection system was applied. These compounds could also be detected by potassium hydroxide (D1). Furazolidone appeared as an orange-brown spot with this reagent. The chromatograms of the heterocyclic compounds (Table II and III) were

* U.V. light at 254 mμ in all cases.

** G. Frederick Smith Chemical Co., Columbus, Ohio.

TABLE II

R_F VALUES OF DRUGS COMMONLY USED IN MEDICATED FEEDS AT 1 μg SENSITIVITY

Compound	*Trivial name*	$R_F \times 100$	*Solvent system No.*
Phenylarsonic acids			
p-Aminobenzenearsonic acid	Arsanilic acid	69	1
		25	7
		35	8
		51	9
p-Ureidobenzenearsonic acid	Carbarsone	54	1
4-Nitrophenylarsonic acid		46	1
3-Nitro-4-hydroxyphenylarsonic acid		36	1
		20	10
		45	12
Nitrofuraldehydes			
3-(5-Nitrofurfurylideneamino)-2-oxazolidone	Furazolidone	84	2
		72	8
		33	9
		28	11
5-Nitro-2-furaldehyde acetylhydrazone	Nihydrazone	64	2
1-Ethyl-3-(5-nitro-2-thiazolyl)-urea	Nithiazide	50	2
5-Nitro-2-furaldehyde semicarbarzone	Nitrofurazone	43	2
		38	11
Heterocyclic compounds			
Thiodiphenylamine	Phenothiazine	81	3
2-Sulfanilamidoquinoxaline	Sulfaquinoxaline	68	3
Hexahydropyrazine	Piperazine	58	3
1-(4-Amino-2-*n*-propyl-5-pyrimidinylmethyl)-2-picolinium chloride hydrochloride	Amprolium	16	3
		35	6
		87	7
Nitrophenyl compounds			
m,m'-Dinitrophenyl disulfide	Nitrophenide	95	4
3,5-Dinitrobenzamide		73	4
		80	10
3,5-Dinitro-*o*-toluamide	Zoalene	73	4
4,4'-Dinitrocarbanilide and 2-hydroxy-4,6-dimethylpyrimidine	Nicarbazin	25*	4
		50*	
Aryl esters			
Diacetate of 3,4-bis-(*p*-hydroxyphenyl)-2,4-hexadiene	Dienestrol diacetate	60	5a**
		57	5b**
		77	11
		73	12
Methyl 4-acetamido-2-ethoxybenzoate	Ethopabate	25	5a
		15	5b
		80	6
Miscellaneous			
Acetyl-(*p*-nitrophenyl)-sulfanilamide		70	10

* R_F 25 is of a freshly prepared nicarbazin standard; R_F 50 is of a nicarbazin standard approximately 2 weeks old.

** 5a is Silica Gel GF on flexible plastic sheet; 5b is Silica Gel GF.

subjected to short wave U.V. light (D3) for at least 10 minutes. Phenothiazine then appeared as a reddish-blue spot and sulfaquinoxaline as a dark spot which quenched fluorescence. Piperazine appeared as a yellow spot after copious spraying with vanillin (D8). When the plates were resprayed with potassium hydroxide (D1), amprolium appeared as a red spot. Sulfaquinoxaline could be detected by *p*-dimethylaminobenzaldehyde (D6) or by barium diphenylamine sulfonate (D2). Piperazine could be detected with β-naphthoquinone-4-sulfonic acid (D5).

TABLE III

DETECTION SYSTEMS FOR TLC OF DRUGS USED IN MEDICATED FEEDS

Class of compounds	*Detection system*
Phenylarsonic acids	D4 or D1
Nitrofuraldehydes	D2 followed by D1
Heterocyclics	D3 followed by D8 followed by D1
Nitrophenyl compounds	D2
Aryl esters	D3

Nicarbazin is a molecular addition compound which decomposes upon aging. The R_F values reported (Table II) represent a standard prepared daily and a standard about 2 weeks old. Potassium hydroxide (D1) was used to distinguish zoalene from 3,5-dinitrobenzamide; zoalene appeared as a yellow spot and the 3,5-dinitrobenzamide appeared as a pink spot. These compounds could not be separated by TLC because of their structural similarity. Nicarbazin could also be detected with this detection system (D1).

Table IV lists the systems which can be used for the detection of ingredients of the common mixtures. The 3-nitro-4-hydroxyphenylarsonic acid of mixture D appeared as a bright yellow spot after it was heated at 100° for 20 min. Dienestrol diacetate appeared as a dark spot after it was copiously sprayed with the antimony trichloride reagent (D7).

Mixture E is a multicomponent drug known commercially as "Unistat". 3-Nitro-4-hydroxyphenylarsonic acid was sometimes difficult to see after it was sprayed with detection agent D2. When the plate was resprayed with potassium hydroxide (D1), the arsonic acid was visible as a yellow spot.

TABLE IV

DETECTION SYSTEMS FOR TLC OF DRUG MIXTURES USED IN MEDICATED FEEDS

Common mixtures	*Detection system*
A Ethopabate; amprolium	D3*
B Amprolium; arsanilic acid	D1
C Furazolidone; arsanilic acid	D1
D Dienestrol diacetate; 3-nitro-4-hydroxyphenylarsonic acid	D3 then D7
E 3,5-Dinitrobenzamide; acetyl-(*p*-nitrophenyl)-sulfanilamide; 3-nitro-4-hydroxyphenylarsonic acid	D2
F Dienestrol diacetate; nitrofurazone; furazolidone	D3 then D7

* Adsorbent contains phosphor.

Studies of procedures for separating these drugs from the other ingredients of feeds are now in progress.

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