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Determination of Residual Diaveridine and Sulfaquinoxaline in Hen's Egg, Chicken Plasma and Tissues by High-Performance Liquid Chromatography

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A high-performance liquid chromatographic method for the determination of diaveridine (Dv) and sulfaquinoxaline (Sq) in hen's egg, chicken plasma, liver, kidney, heart, spleen, muscle, gizzard, skin and fat was developed using a C_{18} reversed-phase column and simple mobile phases. The pretreatments of Dv and Sq in such biological materials were classified into six groups. Detection limits were 0.02—0.04 ppm for Dv and 0.01—0.03 ppm for Sq. Average recoveries of Dv and Sq from egg and plasma were above 77%, and those from all other tissues ranged from 53 to 78%. The method was applied for residue analysis of Dv and Sq in egg yolk and albumen.

Keywords——residue analysis; diaveridine; sulfaquinoxaline; egg yolk; egg albumen; high-performance liquid chromatography

A method for determining residual concentrations is required when a diaveridine (Dv) and sulfaquinoxaline (Sq) antibacterial mixture formulation is used for chickens. In general, Dv is assayed biologically and cannot be measured simultaneously with Sq, while Sq in biological fluids can be determined by thin-layer chromatography (TLC),[1] but these methods are less sensitive and less specific than high-performance liquid chromatography (HPLC), which, however, has not been used for the determination of Sq in real biological fluids.[2]

In the present work, we developed a specific, rapid and sensitive HPLC method for the simultaneous determination of Dv and Sq levels in hen's egg albumen, chicken plasma and tissues, and Dv and Sq levels in hen's egg yolk were analyzed individually.

Experimental

Apparatus——An ALC/GPC 244 liquid chromatograph equipped with a Model 440 ultraviolet (UV) absorbance detector (254 nm, Waters Assoc., Milford, U.S.A.) and a Chromatopac C-R1A (Shimadzu Seisakusho Co., Ltd.) was used. A reversed-phase column, 30 cm × 4 mm I.D. packed with Nucleosil $10C_{18}$, was obtained from Nagel. The precolumn used was commercial RP-18 (3 cm × 4.6 mm I.D.). The column temperature was the ambient temperature (22—25°C) and a 10—50 μl portion of sample solution was injected with a Waters intelligent sample processor (WISP, type 710A). All purging and assay work were done at a constant flow rate of 1.0 ml/min. The mobile phase was 0.05 M phosphate buffer: acetonitrile (3: 1, methods I—IV; 77: 23, method V, VI) mixture used after filtration through a 0.4 μm microfilter (Fuji Film Co., Ltd.).

Drugs and Solvents——Diaveridine [Dv: 2,4-diamino-5-(3′,4′-dimethoxybenzyl)pyridine] and sulfaquinoxaline [Sq: N^1-(2-quinoxalyl)sulfanilamide] were used as received from Burroughs Wellcome and Co. Acetonitrile of HPLC solvent grade (Kanto Kagaku Co., Ltd.) and other solvents of special grade were used without further purification.

Standard Solutions——About 50 mg of Dv or Sq was accurately weighed into a 50-ml volumetric flask. Dv was dissolved in 50% acetonitrile (acetonitrile: water = 1: 1, v/v) and Sq in 80% acetonitrile. These solutions were diluted with 50% acetonitrile to the desired concentrations and were stable for at least 1 week at room temperature.

Procedure——The procedures for assays of Dv and Sq in hen's egg yolk and albumen, chicken blood plasma and tissues were classified into six methods differing in the pretreatment for clean-up.

Method I: Dv in egg yolk: Egg yolk was separated from egg albumen, and 5 g was weighed into a 50 ml centrifuge tube. Fifteen milliliters of ethyl acetate and 0.5 ml of 1 N NaOH were added to the tube, and the mixture was shaken vigorously for 5 min. After centrifugation,[3] the extract was decanted into another 50 ml centrifuge tube. Ten milliliters of ethyl acetate was added to the lower yellow gelatinous phase, and the mixture was again shaken vigorously for 5 min. After centrifugation, the upper layer was decanted and combined with the first extract. The combined extract was mixed with 10 ml of 0.1 M HCl:

AcONa buffer (pH 2.0) and the mixture was shaken vigorously for 5 min. After centrifugation, the organic phase was removed by aspiration. Exactly 8 ml of aqueous phase was transferred to a 20 ml centrifuge tube. The pH of the aqueous phase was adjusted to above 12 by adding 2 ml of 2 N NaOH, then 6 ml of chloroform was added, and the mixture was shaken vigorously for 5 min. After centrifugation, 5 ml of the chloroform phase was transferred to a 10 ml conical centrifuge tube and evaporated[4] to dryness under reduced pressure at room temperature. The residue was dissolved[5] in 200 μl of 50% acetonitrile and filtered through a 0.4 μm microfilter.[6] Ten microliters of the filtrate was injected onto the HPLC column.

Method II: Sq in Egg Yolk: Egg yolk was separated from egg albumen, and 5 g was weighed into a 50 ml centrifuge tube. Fifteen milliliters of ethyl acetate was added to the tube, and the mixture was shaken vigorously for 5 min. After centrifugation, the extract was decanted into another 50 ml centrifuge tube. Ten milliliters of ethyl acetate was added to the lower yellow gelatinous phase, and the mixture was again shaken vigorously for 5 min. After centrifugation, the upper layer was decanted and combined with the first extract. The combined extract was mixed with 10 ml of 0.01 M Na_2CO_3, and the mixture was shaken vigorously for 5 min. After centrifugation, the organic phase was removed by aspiration. Exactly 8 ml of aqueous phase was transferred to a 20 ml centrifuge tube. The pH of the aqueous phase was adjusted to 4.5—5.0 by adding 3 ml of 0.1 M HCl: AcONa buffer (pH 2.0), then 6 ml of ethyl acetate was added and the mixture was shaken vigorously for 5 min. After centrifugation, 5 ml of the organic phase was transferred to a 10 ml conical centrifuge tube and evaporated[4] to dryness under reduced pressure at room temperature. The residue was dissolved[5] in 200 μl of 50% acetonitrile and filtered through a 0.4 μm microfilter.[6] Ten microliters of the filtrate was injected onto the column.

Method III: Dv and Sq in Egg Albumen: Egg albumen was separated from egg yolk and homogenized. About 5 g of homogenate was weighed into a 50 ml centrifuge tube. The homogenate was adjusted to pH 6.0 by adding 1 ml of 1 N AcOH: AcONa (1: 3) mixture, then 20 ml of chloroform was added, and the mixture was shaken vigorously for 5 min. After centrifugation, 5 ml of the organic phase was transferred to a 10 ml conical centrifuge tube and evaporated[4] to dryness under reduced pressure at room temperature. The residue was dissolved[5] in 100 μl of 50% acetonitrile, and 10 μl was injected onto the column.

Method IV: Dv and Sq in Blood Plasma: One milliliter of plasma was mixed with 1 ml of 0.1 M phosphate buffer (pH 6.0). Six milliliters of chloroform was added and the mixture was shaken vigorously for 5 min. After centrifugation, the aqueous phase was removed by aspiration. Exactly 4 ml of the organic phase was transferred to a 10 ml conical centrifuge tube, and evaporated[4] to dryness under reduced pressure at room temperature. The residue was dissolved[5] in 200 μl of 50% acetonitrile and filtered through a 0.4 μm microfilter.[6] Fifty microliters of the filtrate was injected onto the column.

Method V: Dv and in Liver, Kidney, Heart, Spleen, Muscle and Gizzard: The tissue was minced thoroughly with scissors and about 3 g was weighed into a 50 ml centrifuge tube. Fifteen milliliters of chloroform was added to the tube, and the mixture was homogenized using an Ultra-Turrax (Janke and Kunkel). The homogenizer shaft was washed with 5 ml of chloroform, which was combined with the original homogenate. This suspension was shaken vigorously for 5 min and centrifuged. The extract was decanted into another 50 ml centrifuge tube. The residual homogenate was mixed with 10 ml of chloroform and the mixture was again shaken vigorously for 5 min. After centrifugation, the organic phase was decanted and combined with the first extract. The residual homogenate was mixed with 10 ml of chloroform and 1 ml of 1 N NaOH, and the mixture was again shaken vigorously for 5 min. After centrifugation, the upper aqueous phase was removed by aspiration and the organic phase was decanted and combined with the extract. The combined extract was mixed with 2—6 g of Na_2SO_4 and filtered into a 50 ml round-bottom flask. The filtrate was concentrated to an oily residue in a rotary evaporator (Tokyo Rikakikai Co., Ltd.) at 35±2°C. The residue was dissolved in 2×2.5 ml of hexane and transferred to a 20 ml centrifuge tube. The hexane solution was combined with 10 ml of 0.1 M phosphate buffer (pH 6.6), and the mixture was shaken vigorously for 5 min. After centrifugation, the organic phase was removed by aspiration. Exactly 8 ml of aqueous phase was transferred to a 20 ml centrifuge tube, then 6 ml of chloroform was added, and the mixture was shaken vigorously for 5 min. After centrifugation, the aqueous phase was removed by aspiration. A 5 ml aliquot of the organic phase was transferred to a 10 ml conical centrifuge tube and evaporated[4] to dryness under reduced pressure at room temperature. The residue was dissolved[5] in 100 μl of 50% acetonitrile, and 20 μl was injected onto the column.

Method VI: Dv and Sq in Skin and Fat: Skin and fat samples were pretreated under almost the same conditions as Method V, but the final 50% acetonitrile sample solutions for injection were prepared by filtration with a 0.4 μm microfilter.[6] Forty microliters of filtrate was injected onto the column. In the case of fat tissue, the chloroform extraction from the medium made alkaline by addition of 1 N NaOH was omitted.

Results and Discussion

1. HPLC

HPLC conditions for the separation of Dv and Sq in egg yolk and albumen and plasma were studied using Nucleosil $10C_{18}$ and Nucleosil $10C_8$ with phosphate buffer (pH 5—7):

acetonitrile mixtures as the mobile phase. The optimum system for separation of Dv and Sq was Nucleosil $10C_{18}$/0.05 M phosphate buffer (pH 6.0): acetonitrile (3: 1) mixture. Under these conditions, the retention times of Dv nad Sq were approximately 7.5 and 13.5 min, respectively, and each peak was well resolved from the other peaks in the chromatogram. Figure 1 shows typical chromatograms of Dv and Sq extracts from egg yolk and albumen and blood plasma. However, this mobile phase was not adequate for tissues samples, because the HPLC peaks of Dv and Sq slightly overlapped with those of some tissues components and thus, 0.05 M phosphate buffer (pH 6.0): acetonitrile (77: 23) mixture was used for tissues samples. In this case, the retention times of Dv and Sq were approximately 8 and 15 min, respectively. Figure 2 shows typical chromatograms of Dv and Sq extracts from chicken tissues.

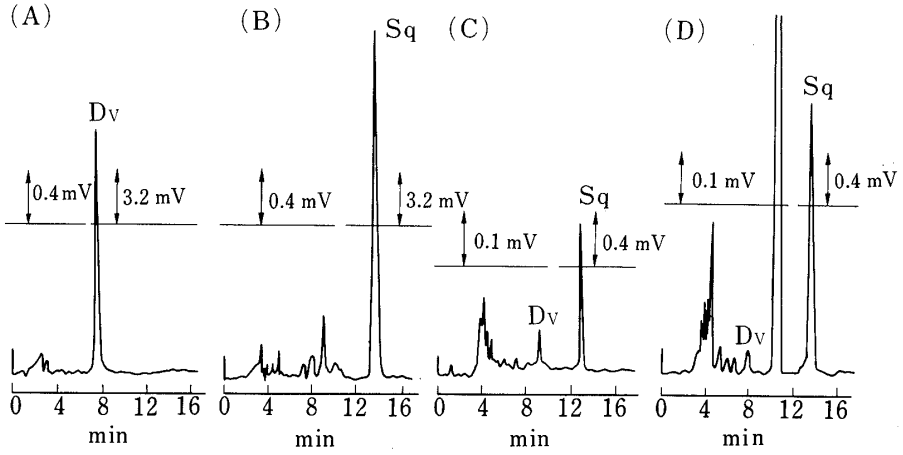

Fig. 1. HPLC Chromatograms of Dv and Sq Extracts from Egg Yolk and Albumen, and Plasma

(A): Dv in egg yolk (3.7 ppm), (B): Sq in egg yolk (8 ppm), (C): Dv and Sq in egg albumen (Dv: 0.04 ppm, Sq: 0.9 ppm), (D): Dv and Sq in plasma (0.2 ppm).

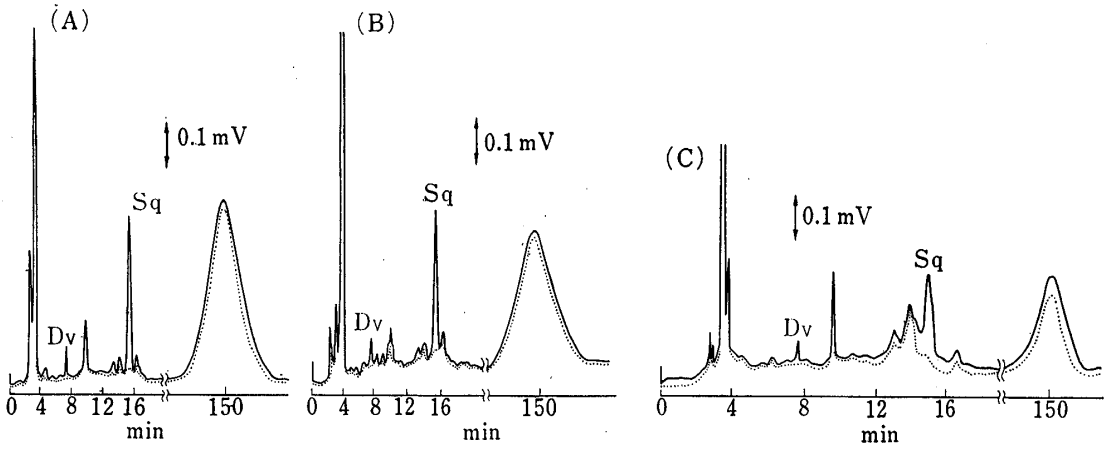

Fig. 2. HPLC Chromatograms of Dv and Sq Extracts from Tissues

(A): Dv and Sq in gizzard (0.1 ppm), (B): Dv and Sq in fat (0.1 ppm), (C): Dv and Sq in liver (0.05 ppm)
——: sample, ·····: control.

The standard curves for Dv and Sq are shown in Table I. A linear relationship between the integrated area or peak height and concentration was observed over the ranges of 10 ng—10 μg per injection for Dv and 1 ng—10 μg per injection for Sq.

2. Clean-up of biological materials

Dv in Egg Yolk——Dv in egg yolk is efficiently extracted with an organic solvent from an alkaline medium. Extractions of Dv in egg yolk with chloroform, ethyl acetate, butyl

Table I. The Standard Curves of Dv and Sq

Compound	Concentration range (ng/injection)	$y=bx+a$		
		b	a	$c.v.$ (%)
Dv	9.8—24[a)]	115	−434	7.60
	16—82[a)]	101	−185	1.19
	82—408[a)]	106	−613	1.03
	101—1010[b)]	0.022	−0.51	2.63
	1010—10100[b)]	0.0022	−0.06	0.64
Sq	1.2—6.1[a)]	643	−624	9.29
	4.0—20[a)]	529	−64	1.15
	20—101[a)]	554	−337	0.73
	99—994[b)]	0.098	−0.09	1.42
	994—9940[b)]	0.0091	1.40	1.80

a) Peak area: x=ng/injection, y=count number.
b) Peak height: x=ng/injection, y=reading on the chart.

Table II. Recoveries of Dv and Sq in Chicken Egg Yolk, Egg Albumen, Plasma and Tissues (*in Vitro*)

Biological material	Dv				Sq			
	Concentration range (ppm)	Recovery (%)	$c.v.$ (%)	n	Concentration range (ppm)	Recovery (%)	$c.v.$ (%)	n
Egg yolk	0.06—10	77	5	44	0.05—21	80	6	50
Egg albumen	0.05— 4	84	8	51	0.05—36	89	5	57
Plasma	0.05— 5	93	10	84	0.05— 8	88	7	52
	2—10	95	8	18	5—25	99	1	18
Liver	0.05— 1	62	13	35	0.05— 1	57	14	35
	1—10	56	3	6	1—10	64	3	6
Kidney	0.05— 1	71	7	23	0.05— 1	63	6	23
	1—10	73	5	20	1—10	65	7	20
Heart	0.05— 1	60	6	7	0.05— 1	67	7	7
	1—10	53	8	9	1—10	70	2	9
Spleen	0.05— 1	75	7	14	0.06— 1	75	8	13
	1—14	74	2	12	1—14	72	7	6
Muscle	0.05— 1	63	7	14	0.05— 1	69	3	14
	1—10	69	7	10	1—10	77	4	10
Gizzard	0.05— 1	74	6	14	0.05— 1	78	4	14
	1—10	72	3	12	1—10	74	2	12
Skin	0.05— 1	63	11	13	0.05— 1	71	8	13
	1—10	72	5	22	1—10	76	4	22
Fat	0.05— 1	53	10	14	0.05— 1	65	9	14
	1—10	66	9	17	1—10	78	6	18

chloride and butyl acetate were examined, and ethyl acetate was adopted for the present purpose, because butyl chloride and butyl acetate caused emulsification, nd chloroform could not be decanted from the egg yolk. Recovery of Dv in egg yolk was examined over the concentration range of 0.06—10 ppm and averaged about 77% (Table II) with a detection limit of 0.02 ppm.

Sq in Egg Yolk——Recovery of Sq in egg yolk was examined by method II in the concentration range of 0.05—21 ppm and averaged about 80% (Table II) with a detection limit of 0.01 ppm.

Dv and Sq in Egg Albumen——Simultaneous extractions of Dv and Sq in egg albumen with chloroform at various pHs were examined. As shown in Fig. 3, both Dv and Sq were extracted very effectively with chloroform at approximately pH 6 [adjusted by addition of

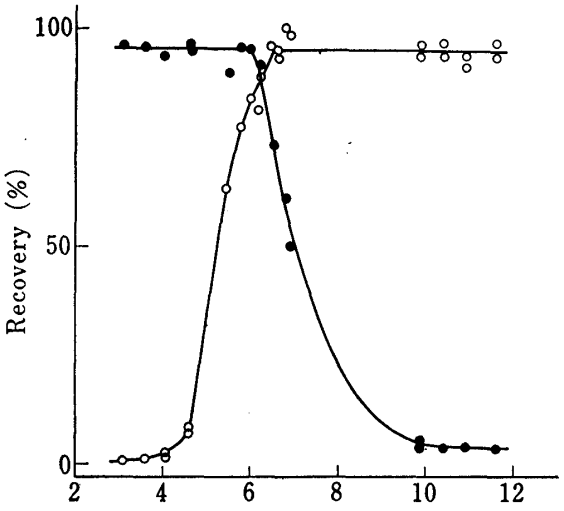

Fig. 3. Relationship between Extraction Recoveries with Chloroform and Controlled pH of Egg Albumen

—○—: D_V, —●—: Sq.

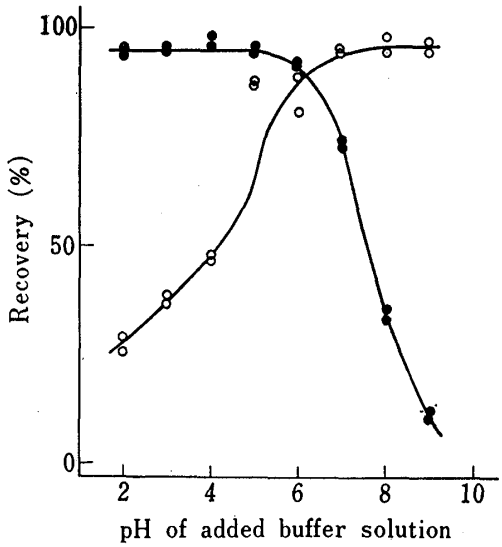

Fig. 4. Effects of pH on Extraction Recoveries of Dv and Sq in Plasma

—○—: D_V, —●—: Sq.

1 N AcOH: AcONa (1: 3) buffer solution]. Recoveries of Dv and Sq in egg albumen were examined in the concentration ranges of 0.05—4 ppm (Dv) and 0.05—36 ppm (Sq), respectively and averaged about 84% and 89% (Table II) with detection limits of 0.04 and 0.01 ppm, respectively.

Dv and Sq in Plasma——One milliliter of plasma containing 1 μg of Dv and Sq was mixed with 1 ml of 0.1 M phosphate buffer (pH 5—9) or 0.1 M HCl: AcOHa buffer (pH 2—4), and the mixture was extracted with 6 ml of chloroform (Fig. 4). Recoveries of Dv and Sq in plasma were examined in the concentration ranges of 0.05—10 ppm (Dv) and 0.05—25 ppm (Sq), respectively, and averaged over 93 and 88% (Table II) with detection limits of 0.04 and 0.03 ppm respectively.

Table III. Residual Concentrations of D_V and Sq in Egg Yolk and Albumen (ppm)

Days after withdrawal[a)]	Dv[a)]				Sq[b)]			
	Yolk		Albumen		Yolk		Albumen	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
0	1.78	0.26	0.19	0.02	1.99	0.34	8.30	1.65
1	1.68	0.18	0.16	0.01	3.06	0.53	6.37	1.09
2	1.30	0.12	0.07	0.05	2.84	0.45	2.07	0.44
3	0.80	0.10	N.D.		2.37	0.76	0.35	0.10
4	0.59	0.14	N.D.		1.74	0.39	0.11	0.03
5	0.46	0.20	N.D.		1.13	0.34	0.08	0.05
6	0.17	0.07	N.D.		0.62	0.29	N.D.	
7	N.D.		N.D.		0.24	0.06	N.D.	
8	N.D.		N.D.		0.15	0.08	N.D.	
9	—		—		0.04	0.03	—	
10	—		—		N.D.		—	
15	—		—		N.D.		—	

a) Dose: D_V–Sq mixture (1: 4), 0.6% drinking water.
b) Each value is the mean (S.D.) of five determinations.
N.D.: not detected, —: not measured.

Dv and Sq in Tissues——Chloroform extraction of Dv and Sq from the tissues except fat was carried out three times with the third extraction done after addition of 1 N NaOH to increase the extraction recovery of Dv. Recoveries of Dv and Sq in tissues were examined in the concentration range of 0.05—10 ppm and overall recoveries ranged from 53 to 78% (Table II) with detection limits of 0.04 ppm (Dv) and 0.03 ppm (Sq). Therefore, recovery factors were used in the calculation of Dv and Sq contents in real tissue samples.

3. Applications

These methods were applied to determine the quantities of residual Dv and Sq in chicken egg yolk, egg albumen, plasma and tissues at 0—15 days after withdrawal of the Dv–Sq mixture (1 : 4). With a dose of 0.6% of a Dv–Sq mixture in drinking water, the residual concentrations of both compounds in egg yolk and albumen were as shown in Table III. At immediately after withdrawal, more Dv was found in the egg yolk than the albumen, while more Sq was found in the egg albumen than the yolk. Dv and Sq disappeared more rapidly from the egg albumen than the yolk. Dv and Sq disappeared from egg yolk after 7 and 10 days and from egg albumen after 3 and 6 days, respectively.

References and Notes

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3) 3000 rpm for 5 min; subsequent experiments were similarly carried out.
4) A Vapour Mix (Tokyo Rikakikai Co., Ltd.) was used.
5) Ultrasonication and a Thero Mix unit were used to ensure dissolution.
6) FR-40 13-00 (Fuji Film) was used.