QUANTITATIVE MICROESTIMATION OF 5-ARYL-6-ALKYL-SUBSTITUTED 2,4-DIAMINOPYRIMIDINES IN BIOLOGICAL FLUIDS AND TISSUES BY ADSORPTION CHROMATOGRAPHY ON SEPHADEX G-25 AND G-10

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A method of adsorption chromatography on columns of strongly "cross-linked" dextran gels Sephadex G-25 and G-10, for the chromatographic separation and quantitative microestimation of 5-aryl-6-alkyl-substituted 2,4-diaminopyrimidines in various biological materials, is described. The chromatographic behavior of these compounds was studied.

Structural analogs of coenzyme forms of folic acid from the group of 5-aryl-6-alkyl-substituted 2,4-diaminopyrimidines (PDAP) are widely used as bacteriostatic and antiparasitic agents [3, 4, 7]. The PDAP are also powerful inducers of anomalies of embryogenesis in mammals at the stage of cleavage and organogenesis [1, 2]. As yet, however, nothing is known of the fate of PDAP in the body, for no methods are available for the quantitative estimation of these compounds in biological materials. The writers have proposed the use of adsorption chromatography on strongly "cross-linked" dextran gels, such as Sephadex G-25 and G-10, for the quantitative determination of PDAP, because these adsorbents have become widely used for the chromatographic investigation of aromatic and heterocyclic compounds [5, 8].

Columns of Sephadex G-25 (1 × 42 cm) and Sephadex G-10 (1.5 × 45 cm) were used to determine the PDAP. All procedures involved in preparation of the gel and column were carried out in accordance with the makers' instructions [6]. The PDAP concentration was calculated from the specific maximum of absorption in the ultraviolet region of the spectrum (Table 1), using calculated coefficients of molar extinction as the starting point. During chromatography, 3-ml samples were collected by means of an automatic collector. The rate of elution was 25 ml/h. The elution volume V_e of the substance was defined as the volume eluting from the time of absorption of half the volume of the applied sample (1 ml) to the middle of the fraction containing the highest concentration of the substance eluted. The coefficient of adsorption distribution of the PDAP was determined by means of Gelotte's formula [5]:

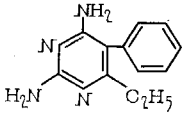
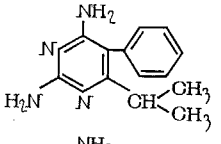
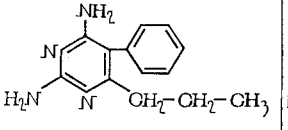
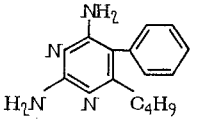
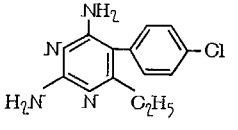
$$K_d = \frac{V_e - V_0}{V_i},$$

where V_e denotes the elution volume of the substance; V_0 the external volume of the column; $V_i = V_a - V_0$. The internal volume of the column V_i is obtained from the difference between the total volume of gel V_a (measured by the acetone method) and external volume V_0 (measured by means of blue dextran with molecular weight 2×10^6) [8].

The principal chromatographic properties of the selected PDAP are given in Table 1. Adsorption was found to increase in the homologous series with an increase in size of the hydrophobic substituent in position 6. This effect was more clearly expressed on the Sephadex G-10 column which has greater ad-

Laboratory of Biochemical Embryology, Department of Embryology, Institute of Experimental Medicine, Academy of Medical Sciences of the USSR, Leningrad. (Presented by Academician of the Academy of Sciences of the USSR S. V. Anichkov.) Translated from Byulleten' Éksperimental'noi Biologii i Medistiny, Vol. 71, No. 6, pp. 122-125, June, 1971. Original article submitted June 4, 1970.

TABLE 1. Chromatographic Mobility of PDAP on Sephadex G-10 and G-25 Columns

Substance	Structural formula	Solubility (in mg/liter)	UV maximum in 1 N HCl solution	UV maximum in 1 N KOH solution	Sephadex G-10							Sephadex G-25					
					pH = 2.0, 0.01 N HCl soln.		pH = 5.0, 0.05 N acetate buffer		pH = 6.0 0.05 M TEB*		pH > 6.0 M < 0.01	pH = 2.0, 0.01 N HCl soln.		pH = 7.3, 0.05 M TEB*		pH = 8.0, 0.05 M TEB*	
					V_e	K_d	V_e	K_d	V_e	K_d		V_e	K_d	V_e	K_d	V_e	K_d
2,4-Diamino-5-phenyl-6-ethylpyrimidine		115,4	273,5	285	125	3,25	129,5	3,4	178,3	5,2	Adsorption	34,5	2,0	46,5	3,0	58,5	4,0
2,4-Diamino-5-phenyl-6-isopropylpyrimidine		41,4	275	285	128,5	3,3	131,7	3,5	178,2	5,2		34,5	2,0	40,5	2,5	58,5	4,0
2,4-Diamino-5-phenyl-6-propylpyrimidine		178,9	276,5	286,5	131,8	3,5	137,5	3,7	192,3	5,7		34,5	2,0	46,5	3,0	58,5	4,0
2,4-Diamino-5-phenyl-6-butylpyrimidine		62,8	276	286	162,5	4,6	164,5	4,7	248	7,8		37,5	2,25	49,5	3,25	70,5	5,0
2,4-Diamino-5-parachlorophenyl-6-ethylpyrimidine (Pyrimethamine)		46,0	271,5	285,5	186	5,95	207	6,3	305	9,9		37,5	2,25	55,5	3,75	70,5	5,0

* TEB denotes tris-EDTA-borate buffer, M = 0.05 (LKB, Sweden).

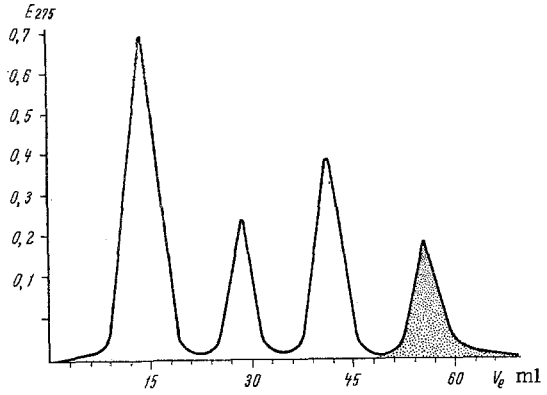


Fig. 1. Adsorption chromatography of freshly prepared albino rat blood serum containing pyrimethamine.

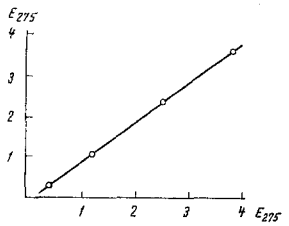


Fig. 2. Nomogram for calculating pyrimethamine concentrations based on total optical density of eluted chromatographic zone. Abscissa, optical density of substance applied to column at λ = 275 nm; ordinate, total optical density of eluted substance.

sorption capacity. The principle of selective chromatography of PDAP on a dextran column is based on the fact that K_d of pyrimethamine and its analogs in M = 0.05 buffer solutions is significantly greater than that of "natural" heterocyclic metabolites of the blood serum (coenzymes, nitrogenous bases, nucleosides, nucleotides, and so on).

Procedure for Determining PDAP in Serum. A sample of 0.2-1 ml of serum containing PDAP was applied to the column equilibrated with 0.05 m tris- or 0.05 M potassium-phosphate buffer (the pH of the buffer was chosen with regard to the type of Sephadex and the chemical composition of the biological material in which the analogs were to be determined). On passage of the serum through the column the high molecular weight components of the serum (including proteins) run out in an external volume of V_0. Later, the low molecular weight metabolites in the internal volume of the column run out with $0 < K_d < 1$. Next, compounds adsorbed on the column and optically active in the ultraviolet region of the spectrum are eluted with $K_d > 1$. Finally, pyrimethamine and its derivatives are desorbed last (Fig. 1). The pyrimethamine concentration was calculated from the total optical density of the eluted zone at the ultraviolet maximum. In this way it was possible to determine as little as 3-5 μg of "free" PDAP in the serum, with an error of determination not exceeding 10%. The optical density of the eluate zones was recorded on a type SF-4A spectrophotometer. A strictly linear relationship applies between the concentration of the soluble form of PDAP contained in the serum and the optical density of the corresponding chromatographic zone, and the concentration of folic acid analogs in the eluted peak can be calculated very simply from the nomogram given in Fig. 2. This must be used because the molar coefficient of extinction of PDAP depends on the ionic composition, ionic strength, and pH of the eluted solution. The concentration of "free" soluble PDAP in subcellular fractions of the cell homogenates can be determined in a similar manner.

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LITERATURE CITED

1. A. P. Dyban, I. M. Akimova, and V. A. Svetlova, Dokl. Akad. Nauk SSSR, 163, 1514 (1965).
2. A. P. Dyban and L. D. Udalova, Genetika, No. 4, 52 (1967).
3. J. J. Burchall and G. H. Hitchings, Brit. Med. J., 3, 578 (1968).
4. J. J. Burchall and G. H. Hitchings, Advances in Enzyme Regulation, 46, 323 (1968).
5. B. Gelotte, J. Chromatogr., 3, 330 (1960).
6. Gel Filtration in Theory and Practice, Uppsala (1968).
7. G. H. Hitchings, Trans. Roy. Soc. Trop. Med. Hyg., 46, 467 (1952).
8. L. Sweetman and W. L. Nyhan, J. Chromatogr., 32, 663 (1968).