Determination of Sulfadiazine and N^4-Acetylsulfadiazine in Biological Fluids by Liquid Chromatography on Silica Gel with an Aqueous Buffer as Mobile Phase

DOUGLAS WESTERLUND ˣ **and AGNETA WIJKSTRÖM**

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Abstract □ Sulfadiazine and N^4-acetylsulfadiazine were determined in biological fluids by the direct injection (plasma after protein precipitation and urine after dilution 100 times) of 20 μl on a silica gel column. The mobile phase was an aqueous citrate buffer (pH 4.0) and UV detection was at 264 nm. Chromatographic selectivity was optimized by the silica gel surface and pH of the mobile phase. Detection limits were ~0.4 μg/ml for sulfadiazine in plasma and ~5 and 7 μg/ml for sulfadiazine and N^4-acetylsulfadiazine in urine, respectively. In quantitations by peak heights relative to an internal standard (sulfamerazine), within-run precisions ($s_{rel\%}$) for sulfadiazine were 1.7 and 4.0% at 40 and 2 μg/ml, respectively, in plasma and 0.76 and 1.7% at 750 and 25 μg/ml, respectively, in urine.

Keyphrases □ Sulfadiazine—N^4-acetylsulfadiazine, determination in biological fluids by liquid chromatography □ Biological fluids—determination of sulfadiazine and N^4-acetylsulfadiazine by liquid chromatography □ Liquid chromatography—determination of sulfadiazine and N^4-acetylsulfadiazine in biological fluids

It has been demonstrated recently that the antibacterial (1) and pharmacokinetic (2–4) properties of sulfadiazine indicate that it is a suitable sulfonamide for combination with trimethoprim in order to utilize optimally the synergistic antibacterial effect that is exerted by such a composition.

Sulfonamides have been determined by tradition in biological material by the colorimetric Bratton–Marshall technique (5) or modifications that partly overcome its lack of specificity. The most important of these was developed by Rieder (6). Recently, the Bratton–Marshall reaction has been automated for determination of sulfisoxazole and sulfamethoxazole in biological fluids (7). Sulfasalazine could be determined in biological samples by direct spectrophotometric measurement at 455 nm (8). Sulfamethoxazole has been determined in serum by spectrofluorometry after extraction with *n*-butyl chloride (9). There have also been published methods in which gas chromatography with both flame ionization (10) and electron capture detection (11), thin-layer chromatography with spectrophotometric scanning (12, 13), and spectrofluorimetric scanning after derivatization with fluorescamine (14), have been used.

Most new methods have utilized HPLC as the separation tool, either after a preliminary extraction (15–17), or by the direct injection of the biological fluid after a preliminary precipitation of proteins and/or dilution (18–24). Nonpolar bonded phases have been used mainly as the support, but underivatized silica gel (15), amino-bonded (18) and cyano-bonded (20) reversed-phase materials have also been utilized. Thus far, no method has been published for determination of sulfadiazine and its main metabolite, N^4-acetylsulfadiazine, in biological material by HPLC. To study in detail the pharmacokinetics of sulfadiazine, the desired limit of determination should be ~1 μg/ml for plasma and ~10 μg/ml in urine.

The present method relies on the direct injection of biological fluid: either plasma after precipitation of proteins or urine after dilution onto the chromatographic column. The support was silica gel with a large specific surface area, and the chromatogram was developed with an aqueous buffer as the mobile phase. The UV absorbance was recorded at 264 nm.

EXPERIMENTAL

Apparatus—The chemicals were weighed on analytical[1] or microanalytical[2] balances. Samples were vortexed[3], and precipitated proteins and tissue were removed by centrifugation[4] at 1000×*g*. The pH was measured[5] when necessary. For column packing a constant pressure gas amplifier pump[6] was used connected to a 30-ml reservoir[7].

The liquid chromatograph consisted of a reciprocating piston pump[8], an injection valve[9] equipped with a 20-μl loop, and a variable wavelength UV detector[10,11] monitored at 264 nm by a recorder[12].

Chemicals—Sulfadiazine[13], N^4-acetylsulfadiazine[13] and sulfamerazine[13] were from approved batches. The buffer substances, citric acid[14], phosphoric acid[14], sodium dihydrogen phosphate[14], and sodium hydroxide[15], were of analytical quality. From stock solutions of the sulfonamides (0.5 mg/ml in 0.01 *M* NaOH), suitable dilutions were made with deionized and doubly distilled water, which also was used for preparation of the chromatographic mobile phases.

Chromatographic Technique—A spherical silica gel[16] with a mean particle diameter of 5 μm and a specific surface area of 500 m^2/g was packed into a precision bore stainless steel column[17] (150 × 4.0 mm) by an upwards slurry packing technique (25). The slurry (1.5-g support in 25 ml of methyl isobutyl ketone[18]) was sonicated[19] for 5 min and forced into the column by methylene chloride at 400 bars. After packing, the column was washed with methanol and water (100 ml each), and the mobile phase was deaerated by vacuum. Equilibrium was obtained after the passage of ~25 ml of mobile phase at a flow rate of 0.85 ml/min. The column void volume (~1.3 ml) was determined by the injection of potassium nitrate.

Sample Preparation—*Plasma*—A 0.5-ml sample of plasma containing sulfadiazine and 125 μl of sulfamerazine (internal standard) solution (1.0 μg/μl) were acidified by the addition of 25 μl of hydrochloric acid. The proteins were precipitated by the addition of 1 ml of 45% ammonium sulfate solution, mixing, and keeping at room temperature for 0.5 hr. After centrifugation at 1000×*g* for 10 min, the clear supernatant was transferred by a Pasteur pipet, equipped with a piece of cotton at the

[1] Analytical balance HL 52, Mettler Instrumente AG, Greifensee, Switzerland.
[2] Microbalance M5 SA, Mettler Instrumente AG, Greifensee, Switzerland.
[3] Fisons Whirlimixer, Fisons Scientific Apparatus, Loughborough, U.K.
[4] FP 10 Centrifuge, Labsystems Oy, Helsinki, Finland.
[5] pH-Meter 26, Radiometer, Copenhagen, Denmark.
[6] Haskel AO 15, Haskel Engineering & Supply Co., Burbank, Calif.
[7] Specac packing bomb, Anal. Accessories Ltd., Orpington, Kent, U.K.
[8] Model 711-47, Laboratory Data Control, Riviera Beach, Fla.
[9] Rheodyne Model 7120, Rheodyne Inc., Berkeley, Calif.
[10] Cecil CE 212, Cecil Instruments Ltd., Cambridge, U.K.
[11] Spectromonitor III, Laboratory Data Control, Riviera Beach, Fla.
[12] Tekman TE 200, Tekman Electronics Ltd., Bicester, U.K.
[13] Department of Organic Chemistry, Astra Läkemedel AB, Södertälje, Sweden.
[14] Merck, Darmstadt, West Germany.
[15] EKA, Bohus, Sweden.
[16] Nucleosil 50-5, Macherey-Nagel & Co, Düren, West Germany.
[17] Handy & Harman, USA.
[18] Fisher Scientific Co., N.J.
[19] Bransonic 220, Branson, Heusenstamm, West Germany.

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tip, to another tube, and 20 μl of the filtrate was injected onto the column. A standard curve (2–40 μg/ml) was obtained by treating 0.5 ml of pooled plasma, 100 μl of sulfadiazine standard solution (0.01–0.20 μg/μl), and 25 μl of sulfamerazine solution (0.5 μg/μl) in the same way as the samples.

Urine—Urine (containing sulfadiazine) (0.5 ml) and 200 μl of sulfamerazine solution (0.5 μg/μl) were diluted to 50.0 ml with water, and 20 μl was injected onto the column after careful mixing. A standard curve (25–750 μg/ml) was obtained by adding 25–750 μl of sulfadiazine and N^4-acetylsulfadiazine solutions (0.5 μg/μl) to 0.5 ml of pooled urine portions, which were treated equivalently to the samples.

Chromatography—Plasma and urine samples were run with the same mobile phase, citric acid buffer (pH 4.0; ionic strength = 0.1; 70 ml of 1 *M* citric acid–83 ml of 1 *M* NaOH were diluted to 1000 ml with water). The flow rate was 0.8–0.9 ml/min and the UV absorbance was recorded at 264 nm. After continuous running the chromatographic performance slowly changed (decreasing capacity ratios and efficiencies), probably because of the adsorption of endogenous compounds onto the support. For column maintenance after each working day, the column was washed first with water (50 ml), then overnight with methanol at a low flow rate (0.1 ml/min), and again just before use with 50 ml of water. By treating in this way, a column could be used for routine analysis (300 plasma or urine samples per week) giving reproducible results for several months.

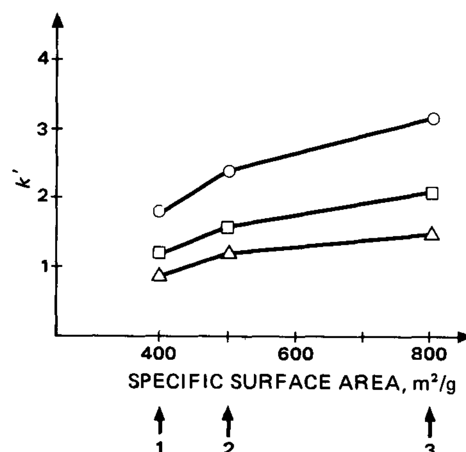

Figure 1—*The dependence of capacity ratios on the support surface area. Mobile phase: citrate buffer (pH 4, μm = 0.1) Column packings: (1) Partisil 5; (2) Nucleosil 50-5;(3) Spherosil XOA-800. Key: (○) sulfamerazine; (□)* N^4*-acetylsulfadiazine; (△) sulfadiazine.*

RESULTS AND DISCUSSION

Chromatography—Sulfadiazine is an amphoteric polar compound which is difficult to extract quantitatively into an organic phase. It is possible to achieve such an extraction, but this necessitates the use of a strongly polar organic medium and/or the addition of ion-pairing counter ions. As a consequence, the obtained extract will contain many coextracted endogenous compounds that may disturb the chromatogram when nonselective UV detection is used. Under such circumstances the introduction of an extraction step only means an unnecessary and roundabout way and time loss in the analytical method. The method was developed to avoid extractions and design the necessary selectivity within the chromatographic system to allow for the direct injection of biological fluids. Preliminary experience with a conventional reversed-phase system (LiChrosorb RP-8 with an acidic methanolic mobile phase) showed significant chromatographic interferences that were hard to control, especially within the retention times for N^4-acetylsulfadiazine.

Underivatized silica gel has been used successfully with aqueous mobile phases for forensic purposes (26, 27) for the determination of the new antidepressant, zimelidine (28), and in studies on ion-pair retention mechanisms (29). A system of this kind was tried for the sulfonamides. It was found at an early stage during the method development that the addition of even small amounts (2%) of organic modifier (*e.g.*, methanol) to the aqueous mobile phase causes the compounds to elute with the front, so neat aqueous buffer solutions had to be used for optimization of the chromatographic system. The actual sulfonamides are only slightly retained by the support in systems of this kind, and it is important to use supports with the largest possible effective surface area. The capacity ratios of sulfadiazine, N^4-acetylsulfadiazine, and sulfamerazine were nearly linearly related to the nominal specific surface area of three different commercial supports (Fig. 1); a citrate buffer (pH 4) was used as the mobile phase in all cases. The pore diameter of the supports decrease with increasing surface area, and this may account for the retentions obtained for the support with the largest area, since all the area may not be available to the comparatively large eluates in this case.

The chromatographic performance of the 800-m²/g support was, however, not acceptable, giving low efficiency and tailing peaks. Furthermore, it gave coincident retention times for sulfadiazine and sulfamethoxazole, as well as for their N^4-acetylated metabolites. Therefore, the 500-m²/g support was utilized for further studies.

There is not a marked dependence of the retention on pH (Fig. 2). The capacity ratios are generally somewhat larger at higher pH, but a retention reversal between sulfamerazine and N^4-acetylsulfadiazine occurs with an acidic mobile phase. Crommen (29) has shown that in this kind of chromatography the support seems to work as the more unpolar phase relative to the aqueous mobile phase, and demonstrated that the addition of counterions gave an ion-pair effect similar to that obtained in conventional reversed-phase liquid chromatography. It was found that a large hydrophobic counterion (tetrahexylammonium) was required to give an ion-pair effect in this system. At pH 6.5 the capacity ratios increased about three times in the presence of this counterion, but the time needed for column stabilization was long (~15 hr). Later experience also showed that in blank chromatograms from body fluids, endogenous compounds, whose retention times also were influenced by the hydrophobic counterion, interfered with the sulfonamides.

The chromatographic efficiency is typically in the range of 35–45 μm (HETP), while the asymmetry factor (back/front at 10% of peak height) normally is in the range of 1.3–1.8.

The selectivity against trimethoprim is high for all three sulfonamides (α = 2.2) at pH 4 which is used in the bioanalysis, but with this mobile phase it is not as satisfactory relative to other sulfonamides. N^4-Acetylsulfadiazine almost coelutes with sulfamethoxazole (α = 1.07) and N^4-acetylsulfamethoxazole interferes with sulfamerazine (α = 1.05). Changing the pH of the mobile phase, however, will also change the selectivity, since the retentions in this system are dependent on the pKa values of the compounds.

Analysis in Biological Material—The only useful parameter remaining for optimization of the mobile phase for bioanalytical work is pH. The result of such a procedure for urine is demonstrated in Fig. 2. Sulfadiazine is severely interfered with by endogenous compounds at most pH values, but at pH 4 all three actual eluates are resolved from the main interfering compounds. An equivalent optimization for plasma determinations resulted in the same mobile phase pH as for urine. Unfortunately, however, endogenous compounds severely disturbed the N^4-acetylsulfadiazine peak and prevented quantitative determination in plasma by the present method at the very low concentrations of this

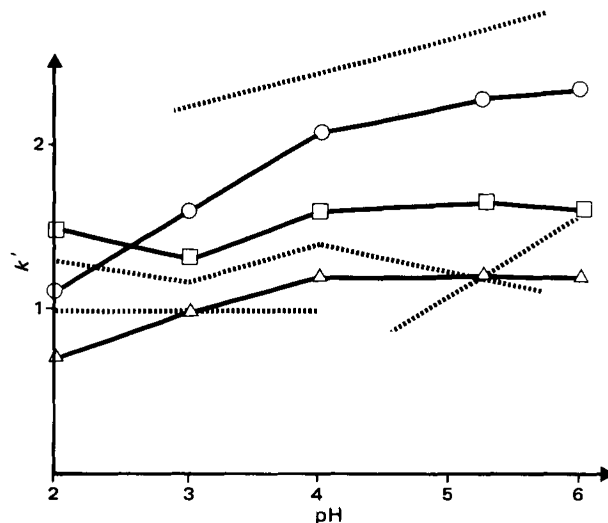

Figure 2—*Optimization of mobile phase pH for urine determinations. Mobile phase: pH ≥4 citrate buffer (μm = 0.1); pH <4 phosphate buffer (μm = 0.1). Key: (△) sulfadiazine; (□)* N^4*-acetylsulfadiazine; (○) sulfamerazine (internal standard); (·····) endogenous peaks.*

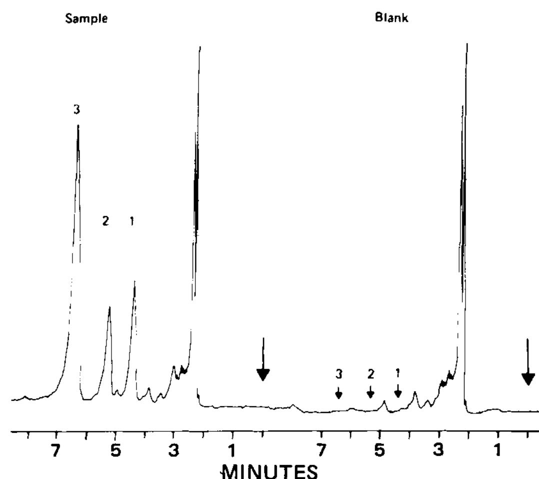

Figure 3—*Chromatograms for determinations in urine (column: 200 × 3.8 mm). Spiked with: (1) sulfadiazine (100 μg/ml); (2) N⁴-acetylsulfadiazine (100 mg/ml); (3) sulfamerazine (400 μg/ml). See text for experimental details.*

compound that normally are encountered in most volunteers and patients. Representative chromatograms from spiked urine and plasma samples are shown in Figs. 3 and 4, respectively.

For plasma determinations, the proteins are precipitated by the addition of ammonium sulfate followed by acidification; this procedure was found to give reasonably clean blank chromatograms and acceptable recoveries. The addition of acetonitrile or methanol for this purpose is not possible, since the chromatographic performance (decreased retention times, the appearance of double peaks, *etc.*) is affected by the presence of an organic solvent in the injected sample. A comparison of peak heights of sulfadiazine (1–15 μg/ml) obtained from plasma samples after protein precipitation by the adopted method and from pure buffer samples, respectively, indicated that the absolute recovery from pooled plasma samples is ~76%. A similar comparison of peak height ratios of sulfadiazine and sulfamerazine, the internal standard, gave congruent standard curves (1–15 μg/ml) from pure mobile phase and plasma, respectively, showing that the recovery of sulfamerazine from plasma is of the same magnitude as for sulfadiazine.

The concentrations of sulfadiazine and N^4-acetylsulfadiazine in human urine are high after the recommended dose (0.82 g × 1 or × 2), even after a single dose, so that a simple dilution of the urine 100 times and the injection of 20 μl of the resultant solution is an adequate procedure for quantitative determinations. The limits of detection for sulfadiazine and N^4-acetylsulfadiazine are ~5 and 7 μg/ml, respectively, under these conditions, corresponding to the injection of 1–1.4 ng of the compounds. It was observed, however, that the selectivity of the chromatographic system may differ between different batches of the support. In some cases the N^4-acetylsulfadiazine peak was interfered with by endogenous compounds also in the urine determinations. In plasma determinations the detection limit for sulfadiazine is ~0.4 μg/ml, *i.e.*, an injected amount of 2.4 ng.

For characterization of the methods, some repeatability studies were performed where the results were calculated in two ways on the same sample: by peak height measurements of sulfadiazine (*i.e.*, no internal standard involved in the calculations) and by peak height ratio calculations (Table I). In accordance with some earlier studies (30, 31), the incorporation of an internal standard, structurally related to the compounds of interest, significantly improves the within-run precisions of the method, presumably by the compensation for the slight changes in chromatographic performance of the column that occur during a working day, and by the elimination of deviations due to small differences in volume between the samples.

Table I—Studies on Quantitative Determinations and Within-Run Precisions

	Added μg/ml of Plasma	$s_{rel\%}$	Mode[a]
Plasma[b]			
Sulfadiazine	40	1.68	IS
	40	2.85	NIS
Sulfadiazine	2	3.97[c]	IS
	2	3.74	NIS
Urine[d]			
Sulfadiazine	750	0.76	IS
	750	2.12	NIS
N^4-Acetylsulfadiazine	750	0.69	IS
	750	1.73	NIS
Sulfadiazine	25	1.67	IS
	25	3.42	NIS
N^4-Acetylsulfadiazine	25	2.46	IS
	25	4.16	NIS

[a] IS, with internal standard; NIS, without internal standard. [b] Internal standard: 25 μg of sulfamerazine/ml of plasma (n = 10). [c] Change of detector sensitivity during the run. [d] Internal standard: sulfamerazine 500 μg/ml and 200 μg/ml, respectively (n = 8).

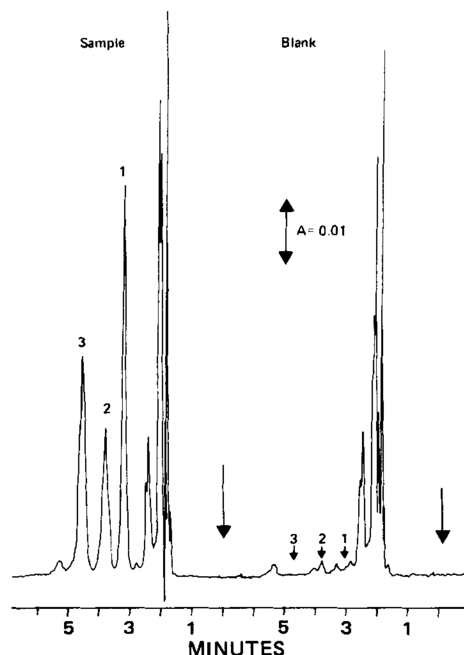

Figure 4—*Chromatograms for determinations in plasma. Spiked with: (1) sulfadiazine (25 μg/ml) in plasma; (2) N⁴-acetylsulfadiazine (15 μg/ml) in plasma; (3) sulfamerazine (20 μg/ml) in plasma. See text for experimental details.*

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Binding of Selected Phenol Derivatives to Human Serum Proteins

JOSEPH JUDIS

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Abstract ☐ The binding of phenol and four of its derivatives to whole human serum and several human serum proteins was investigated. ^{14}C-labeled derivatives were utilized and binding was studied by either equilibrium or dynamic dialysis. Phenol itself was bound least to most of the serum proteins as compared to the derivatives and albumin, and whole human serum exhibited the highest percent binding of the proteins used. Percent binding to albumin and serum paralleled molecular weights of the derivatives, but no definite pattern was observed in ranking the percent binding of the other derivatives to the other serum proteins. Binding constants (K_1, K_2, n_1 and n_2) were determined from Scatchard plots for all the derivatives except *p*-chloro-*m*-xylenol. Phenol was found to have the highest association constant (K_1) and *p-tert*-amylphenol, the lowest. For the entire group of five derivatives and albumin as the protein, a direct, statistically significant correlation was found between percent binding and Hansch π values. No correlation could be found with Hammett σ values. It is concluded that binding of the phenol derivatives to albumin involves primarily hydrophobic bonds.

Keyphrases ☐ Binding—selected phenol derivatives, human serum proteins ☐ Phenol—selected derivatives, binding, selected human serum proteins ☐ Serum—human, binding of selected phenol derivatives to proteins ☐ Derivatives—phenol, selected, binding to human serum proteins

Since the discovery of phenol in 1834 and its introduction to antiseptic surgery by Lister in 1867 (1), both phenol and many of its derivatives have become firmly established as germicidal agents (2, 3). Phenol derivatives, unlike many other germicides, have been shown to be less active in the presence of organic matter (2–4). Blood is a common organic contaminant in materials to be sterilized. Preliminary investigations of the binding of phenol derivatives to human serum proteins were carried out as part of the work in this study on the mechanism of action of phenol derivatives (5, 6). These preliminary studies were expanded to include individual major human serum proteins, percent binding to each, and binding parameters for human serum albumin for a selected group of phenol derivatives.

EXPERIMENTAL

Materials—The phenol derivatives used were obtained with carbon-14 labels[1]. The compounds used and specific activities were as follows: [^{14}C]phenol, 1.57 mCi/mmole; [2,4-^{14}C]dichlorophenol, 0.68 mCi/mmole; [2,4,6-^{14}C]trichlorophenol, 0.68 mCi/mmole; [^{14}C]*p-tert*-amylphenol, 0.27 mCi/mmole; [^{14}C]*p*-chloro-*m*-xylenol, 0.0027 mCi/mmole. Phenol stock solutions were made in distilled water and those of the other derivatives were made with 0.1% NaOH as the solvent. Crystalline human serum albumin and the other human serum proteins were obtained commercially[2] and the whole human serum was of tissue culture quality[3]. All other chemicals used were of reagent grade.

Methods—Dialysis methods (equilibrium and dynamic) were carried out as previously described (7). Radioactivity was determined in a liquid scintillation system using techniques previously described (7, 8). Estimates of binding parameters were calculated using the method of Sandberg (8) and standard Scatchard techniques. All averages were based on a minimum of three replicates.

Table I—Binding of [^{14}C]Phenol and [^{14}C]-*p-tert*-Amylphenol to Human Serum Proteins [a]

Serum Proteins		Ligands Percent Bound[b]	
Fraction	Concentration[c], mg/ml	Phenol	*p-tert*-Amylphenol
Albumin	40.0	48.7 (0.68)	89.1 (0.74)
α Globulin IV-1	1.0	7.9 (0.47)	28.5 (1.92)
α Globulin IV-4	5.0	23.0 (0.63)	46.5 (1.60)
β Globulin III	7.0	3.13 (0.14)	35.2 (0.60)
γ-Globulin II	11.0	8.21 (0.63)	13.0 (0.69)
Human serum	—	52.7 (1.16)	95.3 (0.18)

[a] Each system contained a total of 1.00×10^{-7} mole of [^{14}C]phenol or 1.86×10^{-7} mole of [^{14}C]*p-tert*-amylphenol. [b] The values in parentheses are standard deviations. Data obtained using equilibrium dialysis method. [c] Concentrations used approximate those normally found in human serum.

RESULTS AND DISCUSSION

The values obtained for percent binding to whole human serum and the several serum proteins for the five derivatives studied are listed in Tables I and II. Weakest binding was found with phenol and the latter was bound primarily to albumin. Although phenol was bound to the other serum proteins, the extent was <10% except for α-globulin IV-4. The addition of alkyl groups or halogens to the phenol molecule has been found to increase the latter's germicidal activity (9). It would appear from the results in Tables I and II that alkylation and/or halogenation of phenol also increases the latter's binding affinity for serum proteins. The addition of a tertiary amyl group to phenol increased percent binding of phenol almost twofold (Table I) for albumin and whole human serum and

[1] New England Nuclear Corp.

[2] Nutritional Biochemicals Division of ICN Life Sciences Group.
[3] Difco Laboratories (desiccated TC human serum).

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