Automated Analysis of Chlorthalidone

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Abstract □ Chlorthalidone inhibition of the enzymatic hydrolysis rate of *p*-nitrophenyl acetate by bovine erythrocyte carbonic anhydrase was used as a basis for chlorthalidone determination in plasma and urine. For urinary samples, a completely automated, continuous flow system was developed to extract the samples and perform the enzymatic reaction. Over 100 samples per day could be assayed by one person. The assay had a sensitivity of 0.5 μg/ml and thus could determine urinary concentrations after a therapeutic chlorthalidone dose. To determine plasma concentrations after a therapeutic dose, a manual extraction procedure was used in combination with a second continuous flow system for the enzymatic reaction. This system was optimized to detect the lowest chlorthalidone concentration allowed by the enzymatic inhibition constant and could detect 25 ng/ml.

Keyphrases □ Chlorthalidone—analysis, automated, plasma and urine, carbonic anhydrase inhibition, humans □ Diuretic agents—chlorthalidone, automated analysis, plasma and urine, carbonic anhydrase inhibition, humans □ Automated analysis—chlorthalidone, plasma and urine, carbonic anhydrase inhibition, humans

Previously (1), a method was described in which urinary chlorthalidone was determined by measuring the amount of inhibition of carbonic anhydrase caused by the chlorthalidone. This method was partially automated; the chlorthalidone extraction from the urine was done manually, but the inhibition measurement was automated using continuous flow equipment[1]. The present paper describes a completely automated method by which one person can analyze over 100 urinary samples per day.

Additionally, a partially automated method for plasma sample analysis is described. Since plasma chlorthalidone concentrations after a therapeutic dose (2) are lower than urinary concentrations (1), a method for plasma requires a greater sensitivity than a method for urinary samples. Neither the previous method (1) for urinary chlorthalidone nor the fully automated method described here have sufficient sensitivity for plasma analysis; however, a manual extraction procedure combined with an optimized automated system for the enzymatic reaction was sensitive enough for plasma analysis after therapeutic doses.

EXPERIMENTAL

Reagents for Automated Analysis System—The buffer was 0.1 *M* tromethamine[2], pH 8.0. The enzyme solution contained 40 mg of bovine erythrocyte carbonic anhydrase[2]/liter of buffer plus 0.1% surfactant[3]. The substrate solution was 2.0 m*M* *p*-nitrophenyl acetate[2] in polyethylene glycol 200[4]. The butanol[5] was saturated by shaking five volumes of it with two volumes of glass-distilled, deionized water. Solutions of 0.01 *N* NaOH[5] and 0.1 *N* HCl[5] were used.

Chlorthalidone Standards—A 100-μg/ml stock solution was prepared by dissolving 10.0 mg of chlorthalidone[6] in 10.0 ml of dioxane[7] and diluting to 100 ml with buffer. For urinary standards, the stock solution was diluted volumetrically with normal human urine to concentrations of 0.5, 1.0, 2.5, 4.0, 5.0, 6.0, and 7.5 μg/ml. For plasma standards, the stock solution was diluted with control plasma[8] to concentrations of 0.05, 0.10, 0.15, 0.20, and 0.25 μg/ml. The standards were extracted by the same procedures as were used for the unknown samples.

Automated Procedure for Urine Samples—Figure 1 is a diagram of the continuous flow system[9,10] for urinary chlorthalidone for extraction and analysis. Absorbance chart paper was used for the recorder. The spectrophotometer was used in the inverse mode in which decreases in absorbance are recorded as positive differences on the chart paper. In this mode, the baseline was set to zero with all reagents pumped through their respective lines and the buffer pumped through the sample line. Substrate solution was kept in an ice bath. The standard calibration of the spectrophotometer was adjusted so that a 5.0-μg/ml standard chlorthalidone sample gave a reading of 50 absorbance units.

Samples were assayed using a 40/hr cam with a 3:1 sample–wash ratio. After each 15 samples, the baseline was checked using a buffer sample, and the calibration point was checked using a 5.0-μg/ml standard. For unknown samples, the absorbance units were read directly off the chart and divided by 10 to obtain the chlorthalidone concentration. Samples beyond the linear range, >60 absorbance units, were diluted and reassayed.

Automated Procedure for Plasma Samples—The continuous flow system[9,11] in Fig. 2 was used for plasma samples because it diluted samples less and thus was able to measure lower chlorthalidone concentrations. The spectrophotometer was used in the direct mode. The baseline was adjusted to zero with the buffer pumped through the sample and enzyme lines and the other reagents pumped through their respective lines. The enzyme line was switched to the enzyme solution.

After the large increase in absorbance was recorded, indicating that the enzyme solution had traversed the entire system, the standard calibration of the spectrophotometer was adjusted so that the enzymatic baseline was between 50 and 60 relative absorbance units (*RA*) on the chart. After the enzymatic baseline was established, the sampler was started, and the inhibition of carbonic anhydrase by the samples was determined by measuring the decrease in relative absorbance (ΔRA). Standards were run before and after every 15 unknown samples. The chlorthalidone concentrations of the unknown samples were calculated from a standard ΔRA *versus* concentration curve.

Plasma and urine samples introduced to the carbonic anhydrase and *p*-nitrophenyl acetate reaction decreased the rate of *p*-nitrophenol formed, which decreased the absorbance at 400 nm. In the system for urine samples, these decreases were translated into positive recorder deflections by using the spectrophotometer in the inverse mode. To minimize plasma sample dilution, the spectrophotometer was used in

Table I—Chlorthalidone Standards in Plasma

Chlorthalidone Concentration, ng/ml	ΔRA, Mean ± SD^a
0	0.2 ± 0.6
50	6.3 ± 1.3
100	11.5 ± 0.4
150	15.9 ± 0.8
200	20.7 ± 2.5
250	23.5 ± 2.0
300	28.1 ± 0.8

[a] Quadruplicate samples were extracted from plasma and analyzed.

[1] AutoAnalyzer, Technicon Industrial Systems, Tarrytown, N.Y.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Triton X-405, Rohm and Haas Co., Philadelphia, Pa.
[4] J. T. Baker Chemical Co., Phillipsburg, N.J.
[5] Fisher Scientific Co., Pittsburgh, Pa.
[6] USV Pharmaceutical Corp., Tuckahoe, N.Y.
[7] Mallinckrodt, St. Louis, Mo.
[8] Monitrol, American Hospital Supply Corp., Miami, Fla.
[9] AutoAnalyzer II, sampler IV, proportioning pump III, UV spectrophotometer, and recorder, Technicon Industrial Systems, Tarrytown, N.Y.
[10] AutoAnalyzer heating bath with 40-ft coil, Technicon Industrial Systems, Tarrytown, N.Y.
[11] GCA circulating system, Precision Scientific, Chicago, Ill.

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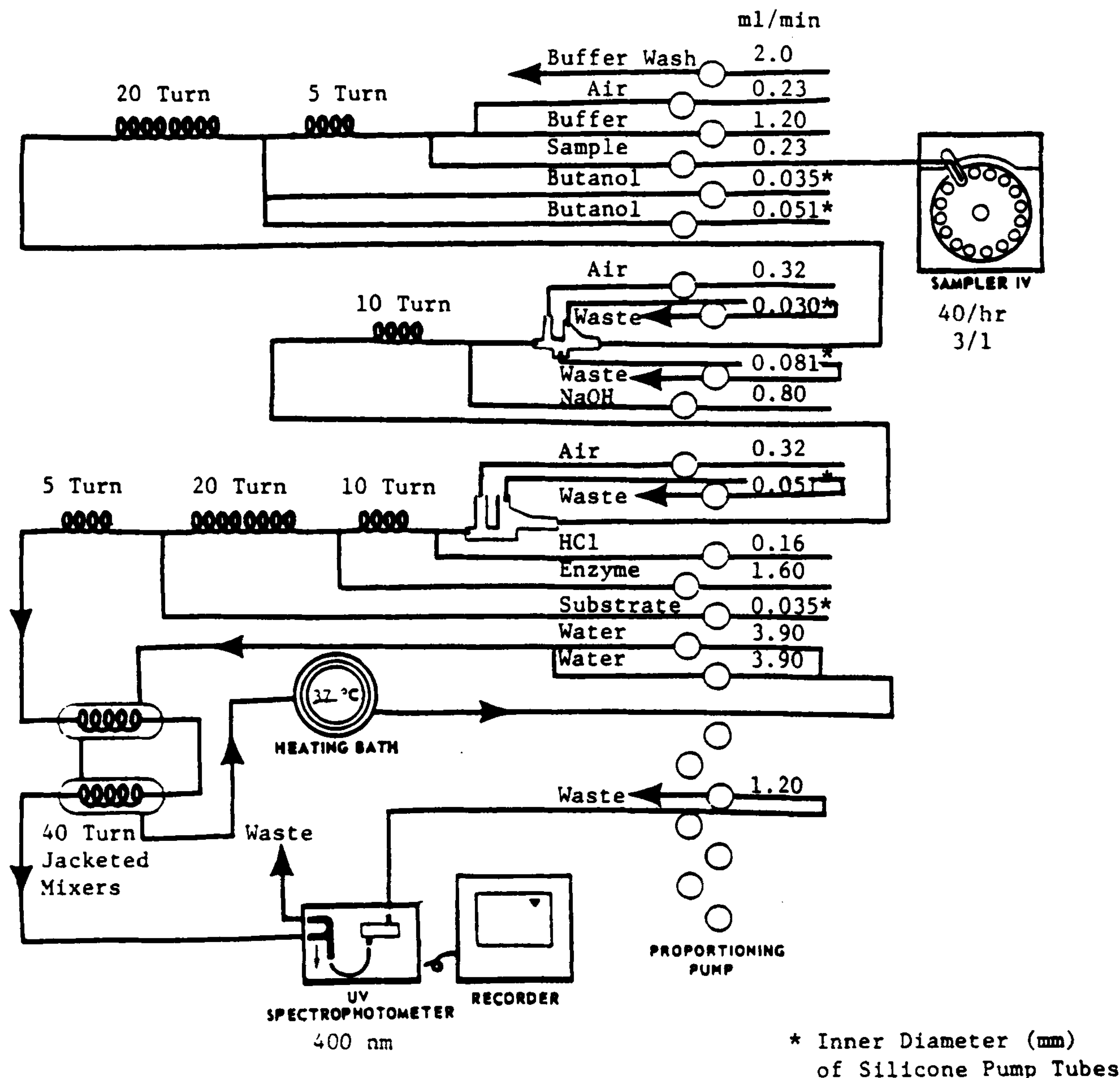

Figure 1—*Continuous flow system for urinary chlorthalidone analysis.*

the direct mode in which the decreases in absorbance (ΔRA) were recorded as shown in Fig. 3.

Extraction Procedure—Plasma was prepared by centrifugation from blood containing either citrate[7] or oxalate as an anticoagulant. Plasma, 1.0 ml, was mixed with 5.0 ml of 0.04 *M* sodium tungstate[7] in 0.09 *M* HCl[5] and extracted twice with 5.0 ml of ether[5]. The ether extracts were combined, and the chlorthalidone was back-extracted with 2.0 ml of 0.1 *M* NaOH[5].

The phases were separated, and the aqueous phase was neutralized with 2.0 ml of 0.1 *M* HCl, buffered with 2.0 ml of 0.1 *M* tromethamine[2] (pH 8.0), and extracted twice with 5.0 ml of ether. The ether extracts were combined and evaporated to dryness, and the residue was dissolved in 2.0 ml of buffer.

Table II—Analysis of Clinical Urinary Samples Containing Chlorthalidone

Sample	Chlorthalidone Concentration, μg/ml: Partially Automated Method (1)	Chlorthalidone Concentration, μg/ml: Fully Automated Method
17	6.96	6.36
25	4.97	4.93
36	3.51	3.52
44	4.50	3.95
52	5.15	5.07
63	11.76	11.70
71	6.67	6.13
78	3.22	3.52
90	13.98	13.40
98	3.92	3.57

RESULTS

Standard Curves—The response of the automated system for urine samples to a series of standard chlorthalidone concentrations in urine was linear up to 6.0 μg/ml. Between 6.0 and 10.0 μg/ml, the response was a nonlinear function of the concentration. Linear regression analysis of urine standards from 0 to 6.0 μg/ml, analyzed 16 times over 2 days, yielded a regression coefficient of $r = 0.999$ and a standard error in chlorthalidone concentration of ±0.12 μg/ml.

Figure 3 shows the response of the system for plasma samples to a series of chlorthalidone standards after extraction from plasma. A plot of ΔRA *versus* chlorthalidone concentration yielded a function that was slightly curved, a phenomenon that had been observed earlier (1). In Table I, the mean and standard deviation values are reported for a series of quadruplicate standards extracted from plasma and analyzed. The sensitivity

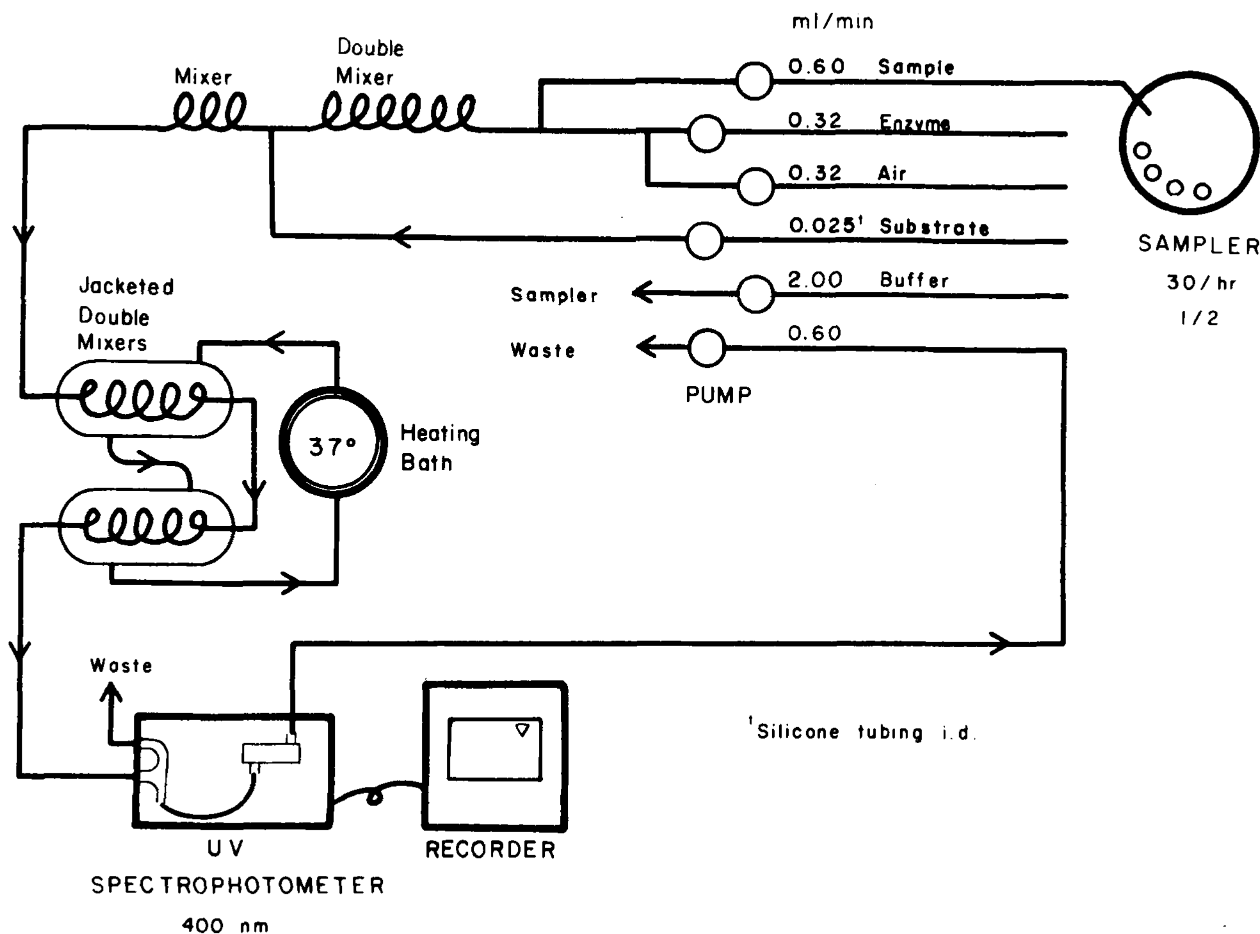

Figure 2—*Continuous flow system for plasma chlorthalidone analysis.*

of the assay was ~25 ng/ml. The extraction procedure efficiency was 50–55% throughout the concentration range.

Clinical Samples—Urinary samples from a subject who had received a therapeutic chlorthalidone dose were analyzed by the previous reported method (1) and by the fully automated procedure described here. Table II shows that both procedures yielded the same results within experimental error. Plasma samples from subjects who had received 100-mg tablets were analyzed, and the concentration–time curves were similar to those obtained by other authors (2, 3) who used GLC methods.

DISCUSSION

Using the fully automated system for urinary samples, one person could analyze 100 or more unknown samples a day. The assay specificity was discussed previously (1). Unknown samples were assayed by both methods, and the results were the same within experimental error. The method is reproducible (±0.12 μg/ml) and is sensitive enough to determine chlorthalidone concentrations after a therapeutic dose (1). Because of these factors, especially speed, this automated method appears to be ideally suited for the analysis of a large number of samples, as would be encountered in a clinical bioavailability or bioequivalency study.

An assay for plasma samples needs to be more sensitive than one for urinary samples because plasma chlorthalidone concentrations after a therapeutic dose are lower than urinary concentrations (2, 3). Since this assay depends on carbonic anhydrase inhibition by chlorthalidone, the ultimate sensitivity of the method is dependent on the inhibition constant, $K_I = 3 \times 10^{-7}\ M$ (4). Thus, a 10% decrease in enzymatic velocity (a detectable decrease) would be observed with a chlorthalidone concentration of $3.3 \times 10^{-8}\ M$ in the reaction mixture.

To obtain this maximum sensitivity, the continuous flow system shown in Fig. 2 was designed to minimize sample dilution between the sampler and the substrate addition that starts the reaction. This optimization was discussed in detail previously (5). From the inhibitor concentration at 10% inhibition ($I_{10} = 3.3 \times 10^{-8}\ M$), the chlorthalidone molecular weight (339 g/mole), the dilution in the continuous flow system (0.60 ml sample/1.15 ml total), and the extraction efficiency (0.55), an approximate sensitivity of 38 ng/ml was calculated:

$$\text{sensitivity} = \frac{339\ \text{g/mole} \times 3.3 \times 10^{-8}\ M}{(0.60/1.15) \times 0.55} = 38\ \text{ng/ml} \quad \text{(Eq. 1)}$$

The experimental limit as estimated from Table I is 25 ng/ml, probably a result of detecting less than a 10% change in enzymatic velocity. Automation of the extraction procedure for plasma samples was considered but is probably not possible since continuous flow system mechanics involve sampling only part of a desired phase, which would result in lower recovery, greater dilution, and loss of the necessary sensitivity.

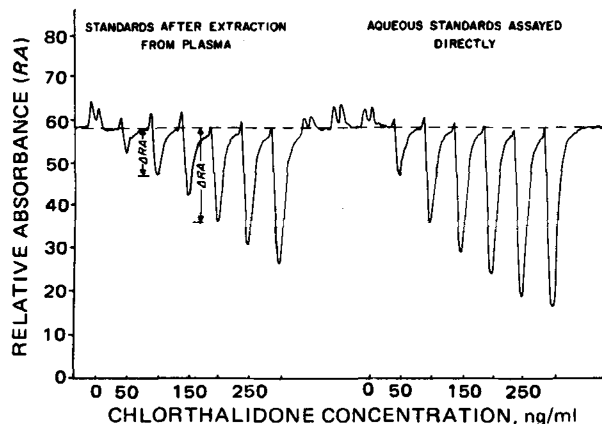

Figure 3—*Typical recording from standard samples analyzed on the continuous flow system (Fig. 2). The samples on the left were extracted from plasma, as described in the text, before introduction into the system. The samples on the right were prepared as known chlorthalidone concentrations in buffer for sampling by the system.*

For clinical studies, especially bioavailability or bioequivalency studies, collection and analysis of urine samples rather than plasma samples may be preferable (6). The distribution of chlorthalidone between plasma and erythrocytes is not instantaneous, so it is difficult to obtain a blood sample and to prepare the plasma quickly enough so that the determined chlorthalidone concentration is the same as the plasma concentration *in vivo* (7). The analysis of urinary samples can be accomplished rapidly with the fully automated system.

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Progestin Permeation through Polymer Membranes IV: Mechanism of Steroid Permeation and Functional Group Contributions to Diffusion through Hydrogel Films

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Abstract □ Hydrogel films were prepared from hydroxyethyl methacrylate, both with (Film II) and without (Film I) 5.25 mole % of ethylene glycol dimethacrylate. Permeation, diffusion, and partition coefficients for progesterone, testosterone, nandrolone, norethindrone, 17α-hydroxyprogesterone, estradiol, and hydrocortisone were determined. A solute permeation model was proposed based on the separation of a domain (B) composed of "bulk-like" water and a domain (A) composed of polymer, interfacial water, and bound water present in the films. The separate contributions from the "pore" and "solution-diffusion" mechanisms to the total permeability were calculated from the model. Steroid permeabilities through Films I and II were analyzed in accordance with this model. Permeation of Film II occurred *via* the solution–diffusion mechanism. Permeation of Film I occurred predominately by the pore mechanism with a small contribution (~20%) from the solution–diffusion mechanism. The latter contribution was dependent on the solubility of the solute within the A domains of the hydrogel film. Functional group contributions to permeation of Film II were ascribed to either steric or hydrogen bonding effects.

Keyphrases □ Progesterone—permeation through hydrogel films, models, structure-activity relationships, steroids □ Hydrogel films—progesterone permeation, structure-activity relationships, steroids, models □ Structure-activity relationships—steroid permeation through hydrogel films □ Models—steroid permeation through hydrogel films

In previous reports from this laboratory (1, 2), the permeation mechanisms of a model hydrophobic drug, progesterone, through poly(hydroxyalkyl methacrylate) films were examined. The importance of film hydration was emphasized. Similar conclusions were drawn by others (3–5) for hydrophilic solute permeation through hydrogel films. Several investigators (2, 4, 5) indicated that, depending on the hydrogel composition, either a "pore" or a "solution–diffusion" mechanism may dominate permeation. For polymers prepared from various comonomers or from hydroxyethyl methacrylate without added cross-linking agents, the pore mechanism dominates. At high concentrations of the cross-linking agent, ethylene glycol dimethacrylate and possibly tetraethylene glycol dimethacrylate, the solution–diffusion mechanism appears to dominate permeation. These results were found for both hydrophobic (2) and hydrophilic (4, 5) solutes.

These conclusions are tenuous without further investigation. One problem is the partition coefficient reported for the hydrophobic solute progesterone (2). This value, which is >100, appears to be inconsistent with a pore mechanism in which transport presumably occurs within water-filled pores or microchannels present in the film. For permeation within these channels, partition coefficients close to one are expected. Hydrophilic solutes in hydrogels generally exhibit partition coefficients close to this value (6).

For this reason, the permeation mechanism of hydrophobic solutes through hydrogel films was examined in greater detail. The permeation characteristics for several steroids that have systematic structural differences were determined in films prepared from hydroxyethyl methacrylate, both with and without 5.25 mole % of ethylene glycol dimethacrylate as a cross-linking agent. The results substantiate previous conclusions concerning the two mechanisms for solute permeation in hydrogel films, provide an explanation for the proposed pore-type permeation mechanism for solutes having high hydrogel–water partition coefficients, and demonstrate the effects of steroid structural differences on permeation rates through hydrogel films.

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