g). On treatment with methanol, this residue deposited white granules of sitosteryl glucoside (50 mg), mp 255°. The identity was confirmed by hydrolysis (dilute hydrochloric acid) of the glycoside to afford the aglycone sitosterol, mp 135–137° (methanol) (GLC–mass spectrometry), and glucose (TLC and co-TLC). Finally, a direct comparison with authentic sitosteryl glucoside[7] showed them to be identical (IR spectrum, melting point, mixed melting point, and co-TLC).

Chromatography (Column III)—The mother liquors from the crystallization of naringenin, fraxetin, and sitosteryl glucoside were combined (11.27 g), adsorbed onto silicic acid[4] (30 g), and chromatographed over silicic acid[4] (300 g) in chloroform. Elution with chloroform–methanol (9:1) gave a fraction (3.01 g), which was treated with methanol (100 ml). The methanol-soluble portion (1.69 g) was chromatographed over silica gel[3] (40 g) in chloroform–ethyl acetate (1:3).

Isolation of Aromadendrin (III)—Elution with chloroform–ethyl acetate (1:3) (1 liter) afforded a residue. On treatment with methanol, this residue gave white granules of aromadendrin (dihydrokaempferol) (14 mg), mp 246° [lit. (14) mp 248°], $[\alpha]_D^{27}$ +22.0° (c 0.5, methanol) [lit. (14) $[\alpha]_D^{27}$ +26°]; UV: λ_{max} (methanol) 228 (log ϵ 3.93) and 293 (3.80) nm; IR: ν_{max} (potassium bromide) 1640, 1590, 1518, 1470, 1440, 1410, 1360, 1288, 1255, 1190, 1170, 1128, 1110, 1088, 1022, 995, 958, 935, 865, 850, 835, 810, 760, and 735 cm^{-1}; mass spectrum (M^+): *m/e* 288 (43%), 259 (55), 165 (22), 153 (100), 136 (36), 134 (41), 107 (51), 77 (12), and 69 (18). This compound was identified as aromadendrin (dihydrokaempferol) by direct comparison[8] (UV, IR, and mass spectra, optical rotation, melting point, mixed melting point, and co-TLC).

Isolation of Kaempferol (IV)—Continued elution with chloroform–ethyl acetate (1:3) (1 liter) gave a residue. On treatment with chloroform–methanol, this residue afforded yellow crystals of kaempferol (20 mg), mp 285° [lit. (15) mp 280°]; UV: λ_{max} (methanol) 215 (log ϵ 4.06), 268 (4.00), and 370 (4.09) nm; IR: ν_{max} (potassium bromide) 3320 (br), 1655, 1615, 1570, 1505, 1385, 1320, 1255, 1225, 1175, 1088, 1008, 972, 880, 845, 830, 815, and 795 cm^{-1}; mass spectrum (M^+): *m/e* 286 (100%), 258 (11), 229 (8), 153 (18), 136 (8), 134 (8), 121 (16), 69 (12), and 65 (8). A direct comparison (UV, IR, and mass spectra, melting point, mixed melting point, and co-TLC) with an authentic sample[5] confirmed the identity.

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[8] Dr. W. E. Hillis, CSIRO, Melbourne, Australia.

REFERENCES

(1) B. D. Jackson, "Index Kewensis," vol. I, University Press, Oxford, England, 1895, p. 818.
(2) *Ibid.*, Supplement II, 1904, p. 112.
(3) G. H. M. Lawrence, "Taxonomy of Vascular Plants," Macmillan, New York, N.Y., 1951, pp. 672, 673.
(4) T. Robinson, "The Organic Constituents of Higher Plants," 3rd ed., Cordus Press, North Amherst, Mass., 1975, pp. 107–111, 151–157.
(5) J. B. Harborne, T. J. Mabry, and H. Mabry, "The Flavonoids," Academic, London, England, 1975, pp. 573, 574.
(6) *Ibid.*, pp. 564, 600–617.
(7) W. Karrer, "Konstitution und Vorkommen der Organischen Pflanzenstoffe," Birkhäuser Verlag, Basel, Switzerland, 1958, p. 541.
(8) J. B. Harborne, T. J. Mabry, and H. Mabry, "The Flavonoids," Academic, London, England, 1975, pp. 588–590, 600–617.
(9) *Ibid.*, p. 321.
(10) K. Randerath, "Thin-Layer Chromatography," 2nd ed., Academic, New York, N.Y., 1968, p. 132.
(11) J. A. Lopez, D. J. Slatkin, M. Theiner, P. L. Schiff, Jr., and J. E. Knapp, *Phytochemistry*, **13**, 300 (1974).
(12) R. Stevens, "Dictionary of Organic Compounds," 4th ed., 5th and Cumulative Supplement, Oxford University Press, Oxford, England, 1969, p. 677.
(13) T. K. Devon and A. I. Scott, "Naturally Occurring Compounds," vol. I, Academic, New York, N.Y., 1975, p. 65.
(14) *Ibid.*, p. 165.
(15) *Ibid.*, p. 126.

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Fluorescamine Use in High-Performance Liquid Chromatographic Determination of Aminocaproic Acid in Serum

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Received May 15, 1978, from the *Clinical Laboratories, Methodist Hospital of Indiana, Inc., Indianapolis, IN 46202.* Accepted for publication July 13, 1978.

Abstract □ A sensitive and specific high-performance liquid chromatographic determination of aminocaproic acid in serum is described. ω-Aminocaprylic acid is used as an internal standard. To 10 μl of serum, 10 μl of the internal standard solution and 50 μl of ethanol are added. After centrifugation, a portion of the supernate is evaporated. The residue is dissolved in 750 μl of 50 m*M* dibasic sodium phosphate, and then 250 μl of fluorescamine in acetonitrile (35 mg/100 ml) is added. The reaction mixture is chromatographed using a column of octadecylsilane bonded to silica and 44% acetonitrile in 0.5 m*M* phosphoric acid as the eluent. Quantitation is achieved by monitoring either the absorbance of the effluent at 405 nm or the fluorescence of the compounds with a fluorometer equipped with a flowcell. The method is reproducible, simple, and fast and has a precision of 4.4%.

Keyphrases □ Aminocaproic acid—high-performance liquid chromatographic analysis in serum □ High-performance liquid chromatography—analysis, aminocaproic acid in serum □ Hemostatic agents—aminocaproic acid, high-performance liquid chromatographic analysis in serum

Aminocaproic acid[1] (I), an antifibrinolytic agent, inhibits the conversion of plasminogen to plasmin (1, 2). The chemistry, pharmacological properties, and mechanism of action of I were reviewed earlier (3). Several methods for the determination of I in biological fluids were reported (4–14). Most of these methods were either indirect or required a large sample volume and elaborate extraction and purification steps.

[1] Amicar, Lederle, Pearl River, N.Y.

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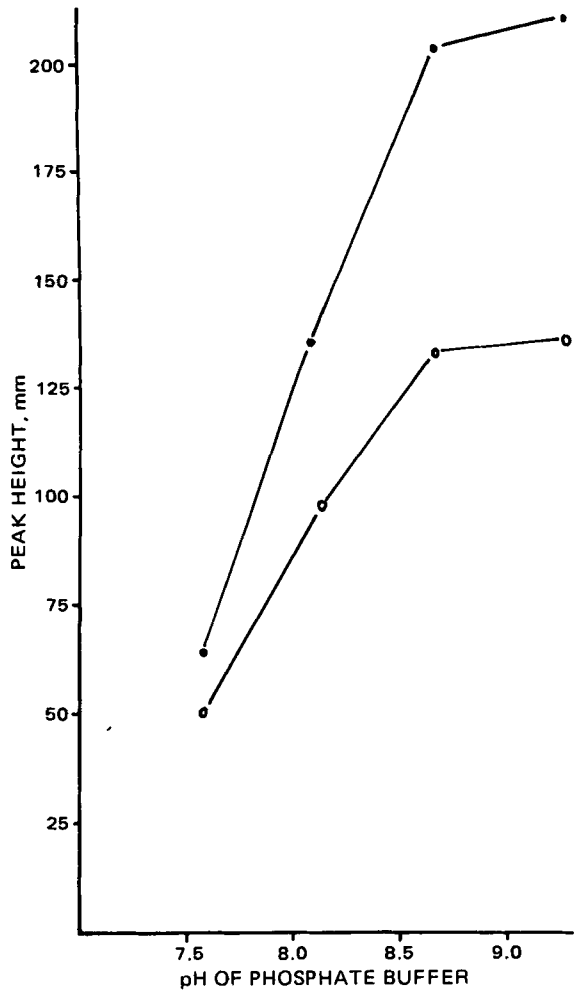

Figure 1—*Effect of change in the pH of phosphate buffer, used in the derivatization of aminocaproic acid (●) and ω-aminocaprylic acid (○) with fluorescamine, on resultant peak heights.*

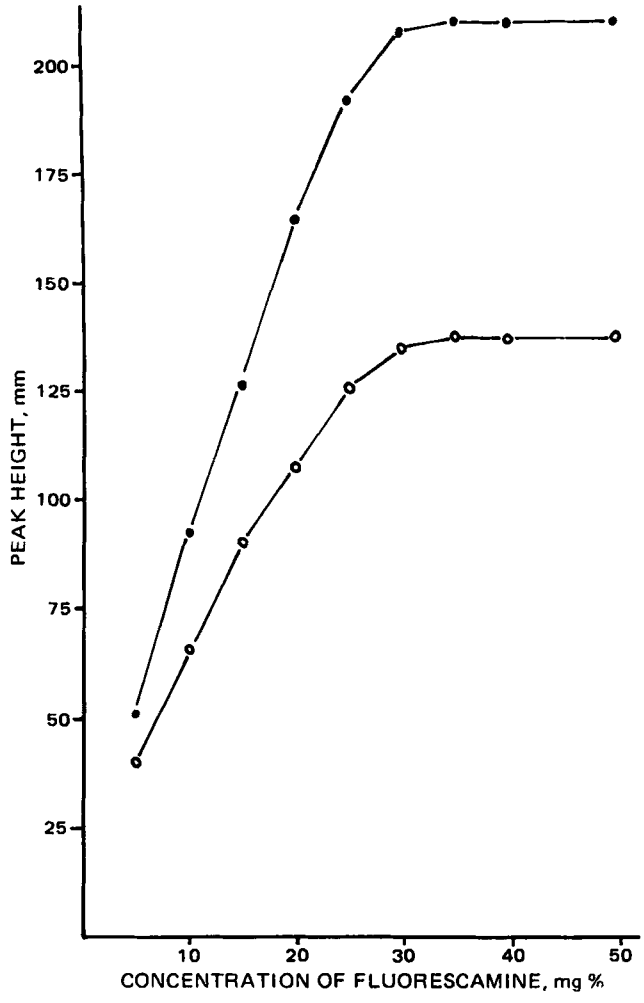

Figure 2—*Effect of change in the concentration of fluorescamine, used in the derivatization of aminocaproic acid (●) and ω-aminocaprylic acid (○), on resultant peak heights.*

Two high-performance liquid chromatographic (HPLC) methods for I in physiological fluids were reported (15, 16). One method (15) utilized postcolumn derivatization of I with ninhydrin and required 2 hr/sample. The fluorometric determination of dansylated I by HPLC (16) required heating for the derivatization reaction and heating of the column to 60°, which is inconvenient in a clinical laboratory.

Fluorescamine[2], a nonfluorescent compound, reacts very rapidly with primary amines to form highly fluorescent pyrrolinone derivatives. While fluorescamine reacts with other nucleophilic functional groups, only primary amines form fluorescent products. These fluorescent derivatives exhibit an absorption maximum at 390 nm and a fluorescence maximum at 475 nm (17). The use of fluorescamine for the formation of fluorescent derivatives of peptides and amines and its use in chromatography were reviewed recently (18). Only three preliminary publications dealt with the HPLC separation of fluorophores formed using fluorescamine. Two reports (19, 20) discussed the separation of the fluorescamine derivatives of dopamine and norepinephrine. The third article (21) was on the separation of some aliphatic diamines.

[2] Fluram, Hoffmann-La Roche, Nutley, N.J.

The need for a rapid, simple, and accurate procedure for the routine determination of I in biological samples and the favorable properties of the reaction of primary amines with fluorescamine led to the present study. The preparation of the fluorescamine derivative of I and the elution and quantitation of the fluorophore by HPLC are described.

EXPERIMENTAL

Apparatus—A modular liquid chromatograph was equipped with an injector[3], a pump[4], a single-wavelength (405-nm) absorbance detector[5], and a filter fluorometer[6]. The fluorometer had a primary filter[7], a secondary filter[8], and a quartz flowcell. The stainless steel column, 3.9 mm i.d. × 300 mm, was packed with octadecylsilane bonded to 10-μm silica[9]. A stainless steel precolumn, 2.1 mm i.d. × 75 mm, contained octadecylsilane bonded to pellicular silica[10].

Reagents—Dibasic sodium phosphate, monobasic potassium phosphate, and phosphoric acid were reagent grade. Acetonitrile was distilled in glass[11].

Fluorescamine Solution—Fluorescamine, 35 mg, was dissolved in

[3] Model U6K, Waters Associates, Milford, Mass.
[4] Model 6000A, Waters Associates, Milford, Mass.
[5] Model 440, Waters Associates, Milford, Mass.
[6] Fluoro-Monitor analyzer, American Instrument Co., Silver Spring, Md.
[7] Corning No. 7-51.
[8] Wratten No. 8.
[9] μBondapak C_{18}, Waters Associates, Milford, Mass.
[10] Co:Pell-ODS, Whatman Inc., Clifton, N.J.
[11] Burdick & Jackson Laboratories, Muskegon, Mich.

Table I—Comparison of Serum Aminocaproic Acid Levels of Patients Determined by HPLC and GLC

Patient[a]	Level, mg/liter HPLC[b]	GLC[c]	Patient[d]	Level, mg/liter HPLC[e]	HPLC[b]
1	84	84	16	ND[f]	ND
2	99	95	17	43	42
3	106	106	18	84	81
4	133	113	19	107	97
5	143	143	20	210	195
6	158	142	21	244	235
7	203	195	22	283	283
8	208	202	23	379	379
9	218	214	24	381	381
10	227	231	25	381	390
11	249	225	26	389	403
12	258	256			
13	263	272			
14	333	356			
15	550	554			

[a] For Patients 1–15 (n = 15), slope = 1.04, r = 0.996, and intercept = −10.7. [b] Using absorbance detector, 405 nm. See text for details. [c] Analyzed by the method of Keucher *et al.* (14). [d] For Patients 16–26 (n = 11), slope = 1.03, r = 0.999, and intercept = −7.1. [e] Using fluorescence detector. See text for details. [f] None detected.

100 ml of acetonitrile. Other concentrations of fluorescamine needed to study the derivatization conditions also were prepared in acetonitrile.

Internal Standard Solution—ω-Aminocaprylic acid[12] (II), 40 mg, was dissolved in 100 ml of water.

Reference Standards—Individual solutions containing 5, 10, 20, 40, and 60 mg of I[12] in 100 ml of water were prepared. Similar concentrations of I in human serum also were prepared.

Procedure—The internal standard solution, 10 μl, was added to 10 μl of serum followed by 50 μl of ethanol. The contents were mixed and centrifuged. A portion of the supernate was transferred to another tube and evaporated under nitrogen at room temperature. The residue was dissolved in 750 μl of 0.05 *M* Na_2HPO_4 (pH 9.3), and 250 μl of the fluorescamine solution was added with mixing.

Chromatographic Conditions—The column temperature was ambient. The mobile phase consisted of 44% acetonitrile in 0.5 m*M* H_3PO_4. The flow rate was 1.8 ml/min. The volume of sample injected was 10–25 μl. The absorbance of the eluted fluorophores was measured at 405 nm with the electrometer set at 0.005 aufs. The fluorescence of the fluorophores was measured using the fluorometer with the relative intensity scale set at 0–10.

RESULTS AND DISCUSSION

Various factors affecting the elution profile were studied. The composition and molarity of the eluting solvent were established first. The optimum concentration of fluorescamine and the pH of the phosphate buffer used in the derivatization of I and II also were determined. These determinations were achieved by injecting equal volumes of solutions containing identical concentrations of I and II prepared under various conditions and then measuring the corresponding peak heights (Figs. 1 and 2). Accordingly, the conditions detailed under *Experimental* for the preparation and elution of the fluorophores were chosen. Under these conditions, the retention times for I and II were 5.0 and 7.8 min, respectively. The fluorophores were stable for several hours. Figure 3 shows typical chromatograms obtained using the absorbance and fluorescence detectors from serum of two patients receiving I and two that did not.

Recovery of known concentrations of I in serum ranging from 50 to 600 mg/liter was studied by using II as a reference standard and comparing peak height ratios to those obtained with aqueous standards of I. The recovery for the concentration range specified was 98.4 ± 2.3% (mean ± *SD*) with a range of 95.1–101.6%.

Triplicate 10-μl samples of serum standards containing 50–600 mg of I/liter were analyzed. The relationship between the concentration of I and the peak height ratio of I to II was linear by either detection method. As little as 10 pg of I injected onto the column could be detected by the fluorescence detector whereas the detection limit for the absorbance detector was 3 ng.

To determine the precision of the method, 20 mg of I was dissolved in 50 ml of serum, divided in small portions, and frozen. Seven analysts

[12] Sigma Chemical Co., St. Louis, Mo.

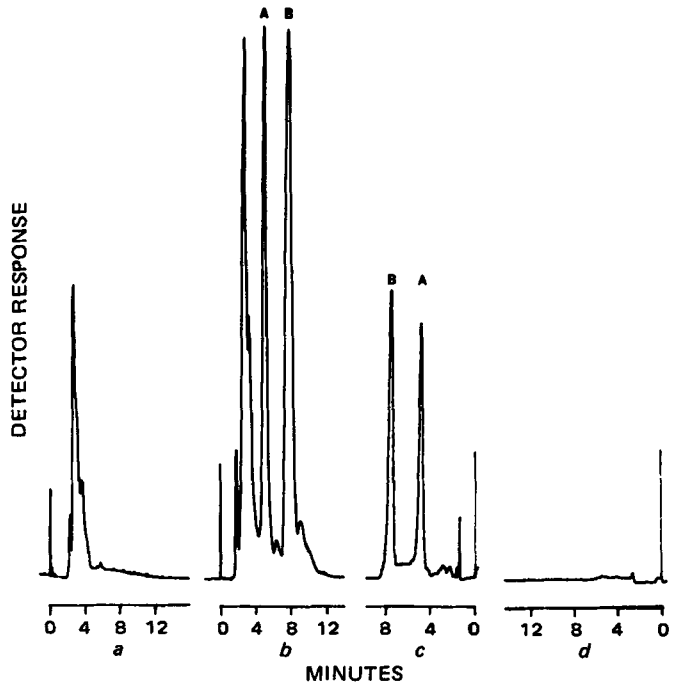

Figure 3—*Chromatograms of serum samples from patients receiving aminocaproic acid (b and c) and patients that were not (a and d), using fluorescence (a and b) and absorbance (c and d) detection systems. Key: A, aminocaproic acid; and B, ω-aminocaprylic acid.*

analyzed 10-μl aliquots of this solution over 75 days. The mean (±*SD*) was 404.3 ± 17.8 mg/liter. This result shows a day-to-day precision of 4.4%. The within-day variation was determined by analyzing one sample five times. The mean was 403.6 ± 4.4 mg/liter.

Fifteen clinical samples were analyzed for I as already detailed and by the method of Keucher *et al.* (14). The results (Table I) indicate an excellent correlation between the two methods. A comparison between the absorbance and fluorescence detectors of the HPLC procedure showed an equally good correlation (Table I).

Serum samples containing several drugs (phenobarbital, phenytoin, ethosuximide, methsuximide, phensuximide, primidone, and theophylline) were analyzed by this procedure to check for possible interference. None of these compounds was detected with either the absorbance or fluorescence detector.

The proposed method for the analysis of I in serum is rapid, sensitive, and easily applied to routine therapeutic monitoring for patients. Five samples of serum can be analyzed in 1 hr. As expected, fluorescence detection is more sensitive than absorbance detection. However, the results indicate that either method could be used satisfactorily in the clinical laboratory.

REFERENCES

(1) N. Alkjaersig, A. P. Fletcher, and S. Sherry, *J. Biol. Chem.*, **234**, 832 (1959).

(2) F. B. Ablondi, J. J. Hagan, M. Philips, and E. C. deRenzo, *Arch. Biochem.*, **82**, 153 (1959).

(3) W. M. Sweeney, *Am. J. Med. Sci.*, **249**, 576 (1965).

(4) A. Sjoerdsma and E. A. Hanson, *Acta Chem. Scand.*, **13**, 2150 (1959).

(5) G. P. McNichol, A. P. Fletcher, N. Alkjaersig, and S. Sherry, *J. Clin. Lab. Med.*, **59**, 7 (1962).

(6) *Ibid.*, **59**, 15 (1962).

(7) Y. Takada, A. Takada, and U. Okamoto, *Keio J. Med.*, **13**, 115 (1964).

(8) N. Wolosowicz, S. Niewiarowski, and K. Czerepko, *Thromb. Diath. Haemorrh.*, **10**, 309 (1964).

(9) N. Wolosowicz and S. Niewiarowski, *Clin. Chim. Acta*, **12**, 555 (1965).

(10) N. Ikekawa, O. Hoshino, R. Watanuki, H. Orimo, T. Fujita, and M. Yoshikawa, *Anal. Biochem.*, **17**, 16 (1966).

(11) F. Markwardt, H. P. Klöcking, and M. Richter, *Pharmazie*, **22**, 83 (1967).

(12) R. W. Zumwalt, K. C. Kuo, and C. W. Gehrke, *J. Chromatogr.*, **55**, 267 (1971).
(13) C. W. Gehrke, K. C. Kuo, and R. W. Zumwalt, *ibid.*, **57**, 209 (1971).
(14) T. R. Keucher, E. B. Solow, J. Metaxas, and R. L. Campbell, *Clin. Chem.*, **22**, 806 (1976).
(15) J. A. Shepherd, D. W. Nibbelink, and L. D. Stegink, *J. Chromatogr.*, **86**, 173 (1973).
(16) R. F. Adams, G. J. Schmidt, and F. L. Vandemark, *Clin. Chem.*, **23**, 1226 (1977).
(17) S. deBernardo, M. Weigele, V. Toome, K. Manhart, W. Leimgruber, P. Böhlen, S. Stein, and S. Udenfriend, *Arch. Biochem. Biophys.*, **163**, 390 (1974).
(18) N. Seiler and L. Demisch, in "Handbook of Derivatives for Chromatography," K. Blau and G. S. King, Eds., Heyden & Sons, Bellmawr, N.J., 1977, pp. 372–376.
(19) K. Imai, *J. Chromatogr.*, **105**, 135 (1975).
(20) G. Schwedt, *ibid.*, **118**, 429 (1976).
(21) K. Samejima, *ibid.*, **96**, 250 (1974).

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Theophylline Protein Binding in Humans

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Received May 2, 1978, from the **Faculty of Pharmacy and the* ‡*Section of Allergy and Clinical Immunology, Department of Pediatrics, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2.* Accepted for publication August 1, 1978.

Abstract □ Theophylline protein binding was 58–82% in serum from six normal adults and 42 asthmatic patients, 1–25 years old, who were given 5 mg of theophylline/kg. The binding range was greatest in young patients, but the proportion of protein-bound drug did not correlate with age. Theophylline protein binding was higher than previously reported. The effect of binding should be considered in patients who do not have optimal bronchodilation from theophylline despite total serum theophylline concentrations of 10–20 μg/ml.

Keyphrases □ Theophylline—protein binding in humans □ Protein binding—theophylline in humans □ Relaxants, smooth muscle—theophylline, protein binding in humans

Drug-protein interactions may influence drug distribution, metabolism, and excretion (1). The extent of theophylline binding to plasma proteins is said to be negligible (2) and is not usually considered when theophylline clearance rates are calculated or when total serum theophylline concentrations are measured.

In this study, theophylline protein binding was measured in serum samples from normal adults and patients with asthma.

EXPERIMENTAL

After informed consent was obtained, six normal adult volunteers not previously treated with theophylline and 42 patients with asthma, 1–25 years old, who had received long-term treatment with theophylline, were given 5 mg/kg po of the drug[1]. Venipuncture was performed 2 hr later. Serum was separated and frozen at −10° for up to 1 month.

Protein binding studies were carried out at room temperature (22°). Serum, 2 ml, was placed in each membrane cone[2]. The cones were centrifuged three times at 1500 rpm for 8–10 min to produce 100 μl of filtrate each time. Theophylline concentrations in the last two ultrafiltrate samples and in an aliquot of retentate were measured by high-performance liquid chromatography[3] (HPLC) (3).

[1] Quibron Elixir, Mead Johnson Canada, Candiac, Quebec, Canada.
[2] Amicon Centriflo cones (CF50), Amicon Corp., Lexington, MA 02173.
[3] Model 8500, Varian Instrument Division, Palo Alto, CA 94303.

Table I—Serum Theophylline Concentrations and Protein Binding Values

Subjects Age, years	*n*	Serum Theophylline Concentration ± *SD*, μg/ml	Theophylline Protein Binding ± *SD*, %	Range, %
Asthmatics, 1–5	10	9.9 ± 4.4	72.1 ± 7.9	58–82
Asthmatics, 6–11	16	9.0 ± 5.2	70.3 ± 4.0	64–76
Asthmatics, 12–25	16	13.4 ± 3.2	70.3 ± 3.2	65–74
Normal adult volunteers, 26–37	6	13.3 ± 2.2	71.8 ± 5.9	66–79

The extent of theophylline protein binding was calculated using:

$$\beta = \frac{D_b}{D_t} = \frac{D_t - D_f}{D_t} \qquad \text{(Eq. 1)}$$

where β represents the fraction of total drug bound to protein, D_b is the amount of drug bound, D_f is the amount of free drug (protein-free filtrate), and D_t is the total amount of drug present (bound plus free). Drug binding was expressed as β%. No corrections for volume change or membrane adsorption were required.

RESULTS AND DISCUSSION

Serum theophylline concentrations and theophylline protein binding values are summarized in Table I. Theophylline protein binding was similar in patients with asthma and in normal adults. The range of binding was greatest in young children, but the proportion of protein-bound drug did not correlate with age.

Previously reported theophylline protein binding values are summarized in Table II. Theophylline was 42–69% bound in plasma from normal adults and in serum from asthmatic mothers who had ingested the drug (4, 5). A similar range was found when theophylline was added to plasma from normal adults who had not received the drug (6). The extent of binding found in both normal adults and patients in the present study was higher, being 58–82%. This result may be due to the various methods of measuring protein binding and theophylline and because either serum or plasma was used, both from dosed patients and from subjects to whose serum drug was added *in vitro* (4–6).

Protein binding has been assumed to be insignificant as far as theophylline distribution, metabolism, and excretion are concerned. Jenne

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