(10) J. Koch-Weser and E. M. Sellers, *N. Engl. J. Med.*, **294**, 311 (1976).
(11) K. M. Piafsky and O. Borga, *Clin. Pharmacol. Ther.*, **22**, 545 (1977).
(12) R. Geddes and P. M. White, *Biochem. Pharmacol.*, **28**, 2285 (1979).
(13) C. F. Chignell, *CRC Crit. Rev. Toxicol.*, **1**, 413 (1972).
(14) E. Woo and D. J. Greenblatt, *J. Pharm. Sci.*, **68**, 466 (1979).
(15) E. M. Sellers and J. Koch-Weser, *Biochem. Pharmacol.*, **23**, 553, (1974).
(16) A. C. Bratton and E. K. Marshall, *J. Biol. Chem.*, **128**, 537 (1939).
(17) K. K. Midha, J. K. Cooper, Y. D. Lapierre, and J. W. Hubbard, *Can. Med. J.*, **124**, 264 (1980).

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Stereospecific High-Performance Liquid Chromatographic Analysis of Warfarin in Plasma

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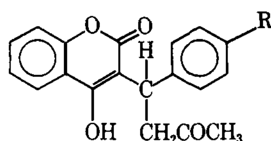
Abstract □ A stereospecific high-performance liquid chromatographic assay has been developed to determine $R(+)$- and $S(-)$-warfarin simultaneously in plasma. The method involved the formation of diastereoisomeric esters, using carbobenzyloxy-L-proline, with subsequent separation using silica as the stationary phase. The method permits characterization of the pharmacokinetics of warfarin enantiomers following administration of racemic drug.

Keyphrases □ Warfarin—stereospecific quantitation in plasma, high-performance liquid chromatography □ Stereoisomers—of warfarin, quantitation in plasma using the racemate, high-performance liquid chromatography □ High-performance liquid chromatography—of warfarin in plasma, quantitation of the stereoisomers using the racemate

Warfarin (I) is administered clinically as a racemic mixture. In humans, $S(-)$-warfarin is five times more potent and is more rapidly eliminated than the R-isomer (1, 2). Consequently, the concentration of each isomer in plasma varies with time within an individual and also between individuals following a dose of racemic warfarin. The response to warfarin is also variable (3). Drugs interact with the isomers differently (4–6). Thus, a more complete understanding of the sources of variability in response to warfarin, and the nature of interactions of drugs with warfarin, requires either giving each isomer separately (a rare clinical procedure) or determining the concentration of each isomer in plasma following administration of the prescribed racemic drug.

Stereospecific analysis of a mixture of enantiomers is difficult. Several specialized analytical techniques, including the synthesis of stable isotopes (pseudo-racemates) coupled with mass spectrometry (7, 8) and a stereospecific radioimmunoassay (9) have been developed to overcome this difficulty.



I R = H
II R = F

Chromatographic separation of enantiomers is possible if a diastereoisomeric relationship is established between them either through the use of chiral solvents (10–12) or derivatization with a suitable chiral reagent (13–15). This paper describes a simple method for the quantitative estimation of the isomers of warfarin in plasma using carbobenzyloxy-L-proline to form diastereoisomeric esters, which can be separated by high-performance liquid chromatography (HPLC) using silica as the stationary phase.

EXPERIMENTAL

Reagents and Materials—Racemic warfarin was obtained from its sodium salt[1] by precipitation with 0.1 M HCl. The dried material was recrystallized from absolute ethanol. $R(+)$- and $S(-)$-warfarin[2], the internal standard[3] (3-[α-(4′-fluorophenyl-)-β-acetylethyl]-4-hydroxycoumarin; 4′-fluorowarfarin) (II), imidazole[4], dicyclohexylcarbodiimide[5], carbobenzyloxy-L-proline[5], hexane (HPLC grade)[6], methanol[7], and ethyl acetate (HPLC grade)[6] were used as supplied. Peroxide-free ether[8] was prepared by passage through a column of activated alumina[9] (45 g, Brockman type 1 alkaline).

Extraction of *RS*-Warfarin—Plasma (0.2 ml) and internal standard (0.1 ml; 0.846 μg/0.1 ml of water) were added to a clean culture tube[10] (16 × 125 mm). The solution was made alkaline with 0.1 M K_2CO_3 (1 ml), shaken manually for 3 min with ether (4 ml), and separated by centrifugation[11] at 3000 rpm for 5 min. After aspiration of the organic layer, the aqueous layer was acidified with 1 M HCl (1.5 ml) and shaken manually for 3 min with ether (6 ml), followed by centrifugation at 3000 rpm for 3 min. The aqueous layer was quickly frozen by immersion in liquid nitrogen (40–60 sec) to allow the organic layer to be decanted into a culture tube whose tip was drawn out to a capacity of 0.2 ml. An antibumping

[1] Sorex, U.K.
[2] Gifts from Endo Laboratories, Inc., U.S.A.
[3] Gifts from Ciba Geigy Ltd., Switzerland.
[4] Sigma Chemical Co., U.S.A.
[5] Aldrich Chemical Co., U.S.A.
[6] Rathburn, Scotland.
[7] Analar; Fisons, U.K.
[8] May and Baker, Dagenham, U.K.
[9] B.D.H., U.K.
[10] Corning, U.S.A.
[11] MSE, Super Minor Centrifuge, No. 533A: MSE, U.K.

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Table I—Chromatographic Parameters for the Carbobenzyloxy-L-prolyl Esters of Warfarin on Various Stationary Phases

Solvent Composition	Stationary[a] Phase	k_S[b]	k_R[c]	R[d]	α[e]
Acetonitrile–water–acetic acid (43:66:1)	Spherisorb 5 ODS	26.2	23.3	1.13	1.12
Ethyl acetate–hexane (15:85)	Spherisorb 5 CN	8.3	10.5	1.44	1.26
Ethyl acetate–hexane (25:75)	Spherisorb 5 Si	5.0	7.0	2.74	1.40

[a] Column size was 4.6-mm i.d. × 10 cm. [b] Capacity factor for the carbobenzyloxy-L-prolyl ester of *S*-warfarin. [c] Capacity factor for the carbobenzyloxy-L-prolyl ester of *R*-warfarin. [d] Resolution factor. [e] Selectivity.

granule was added to the organic layer, which was evaporated to dryness on a heating block at 45° under a stream of nitrogen. The inside of the tube was washed several times with small (100–200 μl) volumes of ether, with vortexing and evaporating to dryness between additions.

For each day of analysis a calibration curve was prepared using the above extraction procedure on blank plasma (0.2 ml) spiked with an aqueous solution (0.1 ml) containing 0.2–2.0 μg of *RS*-warfarin and internal standard (0.8 μg, 0.1 ml).

Derivatization—The derivatization procedure involves esterification with carbobenzyloxy-L-proline using imidazole as catalyst and dicyclohexylcarbodiimide as the condensing agent. To the plasma extract in the modified culture tubes was added carbobenzyloxy-L-proline (10 μl; 100 mg/ml of acetonitrile), imidazole (10 μl; 1 mg/ml of acetonitrile), and dicyclohexylcarbodiimide (10 μl; 100 mg/ml of acetonitrile). The mixture was vortexed for ~10 sec, during which time a precipitate formed. The reaction was allowed to proceed for 2 hr. The tubes were centrifuged at 3000 rpm for 5 min, and aliquots (3–10 μl) of the supernatant were analyzed by HPLC.

High-Performance Liquid Chromatography—For HPLC discrimination of the diastereoisomers, a pump[12], fitted to a stainless steel column (250 mm × 5 mm-i.d.) packed with silica[13] was used with ethyl acetate–hexane–methanol–acetic acid (25:74.75:0.25:0.4) as mobile phase, at a flow rate of 1 ml/min (~600 psi). Test samples were applied by means of an on-column injector, and the effluent was monitored at 313 nm (0.005 AUFS) using a UV detector[14]. All analyses were performed at ambient temperature. Chromatographic tracings were obtained on a strip chart recorder[15] at a chart speed of 1 cm/2 min. The concentrations of the isomers in the unknown samples were calculated by reference to the appropriate calibration curves, constructed by plotting the peak height ratios obtained (using either ester of the internal standard) *versus* the known amounts of isomer per sample of plasma.

Synthesis of the Diastereoisomeric Esters—To *RS*-warfarin (100 mg) in a conical flask (10 ml) was added dichloromethane[8] (3.0 ml) and carbobenzyloxy-L-proline (300 mg). This was sonicated to effect solution, and dicyclohexylcarbodiimide (300 mg in 1 ml of dichloromethane) was added. A precipitate formed immediately, but the reaction was allowed to proceed for ~14 hr at room temperature. The mixture was then filtered, and the filtrate was stored in the dark until TLC separation was performed.

Isolation of Diastereoisomers—Prior to sample application, the TLC plates (silica gel GF 254/G60) were chromatographed in methanol, dried, and rerun in the solvent system, ethyl acetate–hexane–acetic acid (30:70:0.2), used for the separation of the diastereoisomers. The filtered solution containing the diastereoisomers was applied as a narrow band ~2 cm from the bottom edge of the plate. The bands were detected using short-wavelength (254-nm) UV light. Identification of the spots was made by chromatographing each ester formed by independently reacting *R*(+)-warfarin and *S*(−)-warfarin with carbobenzyloxy-L-proline. The average time to complete an analysis (15 cm) was 1.5 hr in a nonequilibrated tank. Care was taken not to overload the plate, since this led to band broadening. In the advent of overlap, lines were drawn across the plate a few millimeters above and below where the overlap had occurred. The material contained within this area was used as racemic diastereoisomeric ester. The regions above and below these lines were scraped into tubes labeled *SS* ester and *RS* ester, respectively, and eluted by manually shaking for 2 min with ethyl acetate or dichloromethane (2 ml). The materials were transferred to microcentrifuge tubes (1.5 ml), centrifuged at 12,000×*g* for 1 min, and the supernatant was transferred to a clean vial. An aliquot of each supernatant was chromatographed using the same solvent system used to separate the diastereoisomeric esters. Those fractions chromatographing as either *SS* ester, *RS* ester, or racemic ester were pooled in appropriately labeled vials. The entire process was then repeated using several plates.

A sample of silica gel was taken from an area free from UV-absorbing material and processed as for the diastereoisomers. Mass spectral[16] and IR[17] (solution) analyses were performed on both the silica gel blank and the diastereoisomers.

[12] Altex Model 100A, Anachem Ltd., U.K.
[13] Spherisorb Si 5 μm, Phase Sep., U.K.
[14] Waters Model 440, Waters Associates, U.S.A.
[15] Servoscribe 210, Smiths Instruments, U.K.

Table II—Within-Assay Variability of Peak Height Ratios at Various Amounts of *RS*-Warfarin

Amount of Isomer, μg	Relative Standard Deviation, %			
	*SS*1 [a]	*RS*1 [a]	*SS*2 [b]	*RS*2 [b]
1.0[c]	3.2	3.5	3.25	3.7
0.4[c]	6.3	6.7	5.1	6.6
0.1[d]	9.8	8.4	11.9	11.8

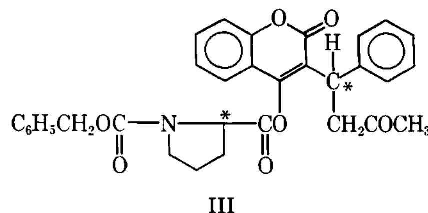
[a] Peak height ratios determined relative to the first eluting ester of the internal standard. [b] Peak height ratios determined relative to the second eluting ester of the internal standard. [c] $n = 6$. [d] $n = 4$.

RESULTS AND DISCUSSIONS

The proposed structures of the carbobenzyloxy-L-prolyl diastereoisomeric esters of warfarin (III) were established (after separation of the diastereoisomers by semipreparative TLC) by low-resolution electron impact (EI) and chemical ionization (CIMS, ammonia as carrier) mass spectrometry (MS) and IR analysis. The chemical ionization MS data, in conjunction with EI studies, verified the predicted molecular weight (CIMS: *m/z* 557, $[M + NH_4]^+$; EI: *m/z* 539, $[M^+]$). These molecular ions were not present in the background sample of silica gel. As expected the MS of the *R*(+)- and *S*(−)-warfarin esters were similar. The IR spectra (chloroform) of the diastereoisomeric esters showed an absorption at 1780 cm^{-1} consistent with vinylic esters (1800–1770 cm^{-1}).

Not all apparently good candidates for the formation of diastereoisomers with *RS*-warfarin gave favorable results. Thus, *N*-acetyl-L-alanine[5] and *N*-acetyl-L-phenylalanine[5] did not form derivatives with *RS*-warfarin using dicyclohexylcarbodiimide as the activating agent. Also, although both *R*(−)-2-methoxy-2-phenylacetic acid[18] and L-menthoxyacetic acid[5] formed derivatives, chromatographic conditions could not be found to achieve resolution of the resultant diastereoisomeric esters on either silica[13] or Spherisorb 5 ODS[13] stationary phases. In contrast, successful derivatization and resolution of *RS*-warfarin was achieved using *N*-acetyl-L-proline (16) and carbobenzyloxy-L-proline (*Z*-L-proline). The latter was eventually chosen because of its higher (>99%) optical purity.

The effectiveness of the derivative for resolution is subject to several criteria: conformational rigidity of groups attached to the asymmetric center [a center as part of a ring system can improve resolution (17)], *e.g.*, *N*-acetyl-L-proline and carbobenzyloxy-L-proline; a minimum distance of 3–4 atoms between the chiral centers of the reagent and solute (18–20); and polar or polarizable groups in close proximity to the asymmetric centers (21). In *R*(−)-2-methoxy-2-phenylacetic acid the asymmetric center is not part of a ring system. Although the asymmetric center of L-menthoxyacetic acid is in a ring sytem, the asymmetric centers of the proposed derivative are six atoms apart. These results suggest that all of the above criteria must be met if adequate resolution is to be effected. The lack of reactivity of the *N*-acyl derivatives of L-alanine and L-phenylalanine may be due to formation of oxazolones (22, 23) when dicyclohexylcarbodiimide is used as the activating agent.



III

[16] Kratos, MS25.
[17] Perkin-Elmer 237 Infra-red Spectrophotometer, Perkin-Elmer, U.S.A.
[18] Fluka, A.G., Switzerland.

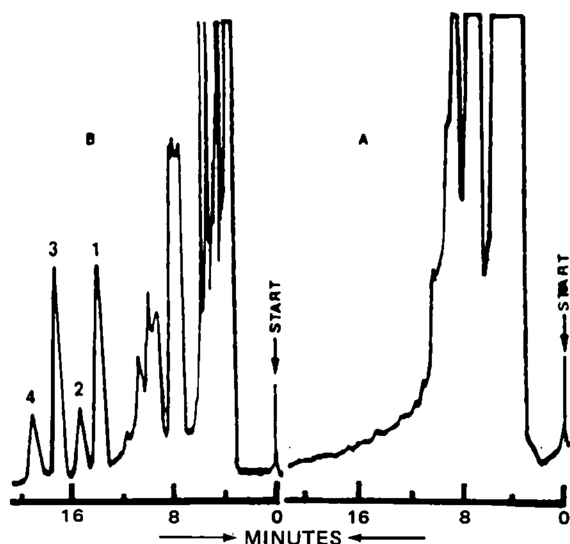


Figure 1—*Chromatograms obtained after derivatization with carbobenzyloxy-L-proline of (A) control human plasma and (B) human plasma spiked with* RS-*warfarin (2 µg). Key: (1)* SS-*warfarin ester; (2) first eluting ester of the internal standard; (3)* RS-*warfarin ester; (4) second eluting ester of the internal standard.*

N,N'-Dicyclohexylurea, a byproduct in the activation of carboxyl functions by dicyclohexylcarbodiimide (24) is soluble only to a certain extent in most organic solvents. To overcome the purification problem caused by the formation of *N,N'*-dicyclohexylurea and *N*-acyl ureas, water-soluble carbodiimides are often used (25). However ester formation with carbobenzyloxy-L-proline in the presence of such water-soluble carbodiimides as 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide methoxy-*p*-toluenesulfonate[5] or 1-(3-dimethyl-aminopropyl)-3-ethyl carbodiimide[5] was inferior to that in the presence of dicyclohexylcarbodiimide. With the first, derivative formation did not occur; with the second, derivatization was incomplete. Even so, as the technique is being developed specifically for analytical purposes, the lack of the option for removal of the urea-type impurities is not a serious disadvantage, because the impurities lack good chromophores at the wavelength (313 nm) used in the analysis of warfarin, so that minimal interference is observed.

Reverse-phase HPLC is popular partly because of the wide variety of stationary phases available and partly because the ability to inject aqueous samples reduces the number of prechromatographic steps required. From preliminary investigations with the stationary phases Hypersil-5 ODS[19], Spherisorb 5 ODS[13], Spherisorb 5 phenyl[13], and Spherisorb 5 CN[13] in the reverse-phase mode only Spherisorb 5 ODS and Spherisorb 5 CN gave sufficient promise to warrant further study. Improved performance was seen, however, when Spherisorb 5 CN was used in the normal-phase mode. Under optimal conditions, using the solvent combinations shown in Table I, Spherisorb 5 ODS showed both low selectivity and resolution. In general, normal-phase chromatography on silica gave the highest selectivity and resolution. Although the maximum difference in selectivity between Spherisorb 5 CN and Spherisorb 5 Si was ~11%, this resulted in a 90% increase in resolution for the diastereoisomeric esters. Addition of acetic acid to the mobile phase when Spherisorb 5 Si is used as stationary phase decreases retention time without adversely affecting resolution or selectivity for the diastereoisomeric carbobenzyloxy-L-prolyl esters (Table I).

The rate of derivatization of *RS*-warfarin (2 mg/ml of ethyl acetate, 10 µl) by carbobenzyloxy-L-proline (100 mg/ml of ethyl acetate, 10 µl) in the presence of dicyclohexylcarbodiimide (100 mg/ml of ethyl acetate, 15 µl), measured by the disappearance of *RS*-warfarin, was followed for 12 hr. The rate is first order with a half-life of disappearance of 3.5 hr. With completion (99%) of the reaction not anticipated before 24 hr, this reaction time was considered too long. In the presence of pyridine (24.5 µl in ethyl acetate) as catalyst, the reaction was complete within 1 hr, and the resultant diastereoisomers remained stable for at least 9 hr. However, the detection of a large peak attributed to pyridine, eluting after the diastereoisomers, limited its use as catalyst because the number of HPLC analyses would be restricted to only two samples per hour. Pyridine absorbs strongly in the 305–315-nm region, which coincides with the wavelength (313 nm) used for detection of the diastereoisomeric esters. Imidazole, which has a lower maximum wavelength of absorption (207 nm), proved to be just as good a catalyst as pyridine; the mechanism of catalysis of esterification is similar (26). Apparently, the presence of a basic nitrogen alone is not sufficient, since triethylamine failed to catalyze the reaction.

[19] Shandon, Southern Products Ltd., U.K.

Table III—Between-Assay Variability of Peak Height Ratios at Various Concentrations of *RS*-Warfarin over a 6-Week Period

Amount of Isomer, µg	Relative Standard Deviation, %			
	*SS*1 [a]	*RS*1 [a]	*SS*2 [b]	*RS*2 [b]
1.0[c]	3.8	3.6	6.0	5.1
0.8[d]	3.1	2.9	5.9	4.2
0.6[d]	6.05	7.8	4.7	6.8
0.4[e]	7.7	7.4	7.5	7.9
0.2[d]	9.0	8.3	9.4	9.9
0.1[f]	13.7	17.1	14.4	13.7

[a] Peak height ratios determined relative to the first eluting ester of the internal standard. [b] Peak height ratios determined relative to the second eluting ester of the internal standard. [c] $n = 26$. [d] $n = 10$. [e] $n = 23$. [f] $n = 16$.

Initially, 4-hydroxycoumarin was chosen as the internal standard; it produced a single ester with carbobenzyloxy-L-proline, eluting just after the *RS*-warfarin ester. However, there was a high degree of variability in the subsequent assay procedure which was identified with an instability of the carbobenzyloxypropyl ester of 4-hydroxycoumarin, probably due to imadazole-catalyzed hydrolysis. The absence of a 3-substituent in 4-hydroxycoumarin (*cf.* warfarin) would make attack of the ester carbonyl more facile, due to lack of steric hindrance, for this ester than for the warfarin ester. Of the many others examined, 4'-fluorowarfarin proved the most suitable with respect to retention time and stability, although being a racemate, diastereoisomeric esters are formed.

A typical chromatogram obtained after derivatization of an ethereal extract from human plasma spiked with *RS*-warfarin and internal standard (4'-fluorowarfarin) is shown in Fig. 1. Identification of the retention times of *RS*- and *SS*-warfarin esters was made by chromatographing each of the esters formed by independently reacting *R*(+)-

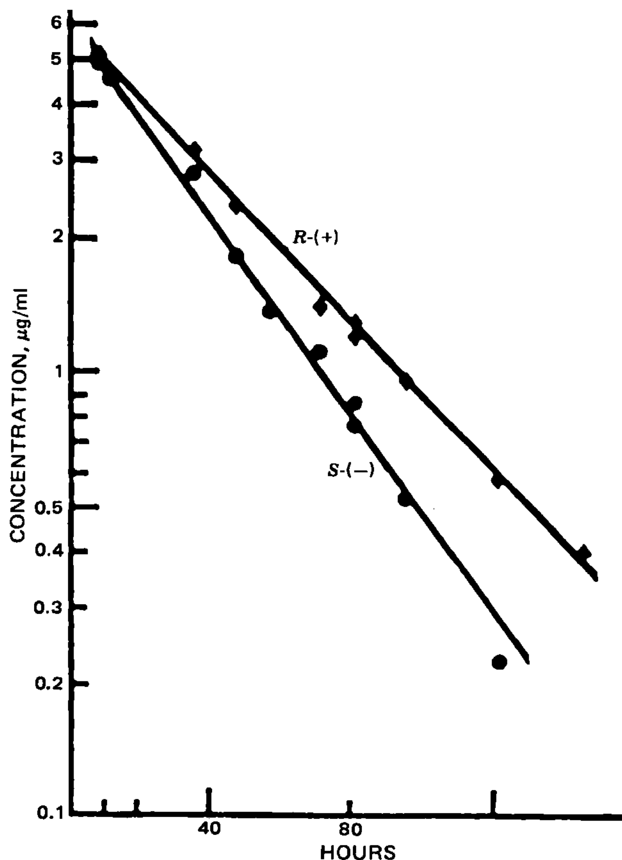


Figure 2—*Plasma concentration–time profile of* R(+)*-warfarin (◆) and* S(−)*-warfarin (●) following oral administration of* RS*-warfarin (1.56 mg/kg) to a human subject.*

warfarin and $S(-)$-warfarin with carbobenzyloxy-L-proline. The $S(-)$-warfarin carbobenzyloxy-L-prolyl ester elutes before the ester of $R(+)$-warfarin, with a separation factor of 3.0. A similar elution order is assumed for the internal standard, but could not be verified because this compound has never been resolved. Repeated analyses with time indicated that the derivatives were stable for at least 48 hr in the final reaction mixture.

The recovery of *RS*-warfarin investigated by taking *RS*-warfarin (0.8 μg) spiked with [^{14}C]*RS*-warfarin through the extraction, derivatization, and HPLC analysis was 70% (n = 3). The major losses occurred during evaporation of the sample, therefore the inside of the tube must be washed to concentrate the extracted material in the tip of the tube.

Linear calibration plots for either isomer ($r^2 > 0.99$) were obtained over the range 0.1–1.0 μg/isomer. As shown in Table II, based on either of the internal standard esters (*RS* or *SS*), the relative standard deviations of the peak height ratios were between 11.9 and 3.2% for 0.1 and 1.0 μg warfarin isomer, respectively. Table III summarizes the results of a similar assessment of between-assay variability for studies performed over a 6-week period using 0.1–1.0 μg of warfarin isomer. Satisfactory precision (<10%) is observed over a fivefold concentration range of warfarin isomers. The results indicate that the assay is accurate and reproducible.

The determination limit of the assay with a 10% coefficient of variation was computed from the expression (27):

$$X_0 = 10\sqrt{V(X_0)}$$

where X_0 is the determination limit at the preselected coefficient of variation, and $V(X_0)$ is the variance associated with X_0. Using the above procedure, the determination limits (10% *CV*) of the UV assay for *S*- and *R*-warfarin are 0.16 and 0.096 μg, respectively, using the first eluting standard. Similar values were obtained when using the second eluting standard. The difference in determination limits of the esters of warfarin may be ascribed to the greater potential for interference between the *SS*-ester of warfarin and the first internal standard, due to changes in retention as a result of column deterioration, or day-to-day variability in eluant composition.

Figure 2 shows the plasma concentration–time profiles for the *R*- and *S*-isomers (using 200-μl plasma samples) following administration of *RS*-warfarin (1.5 mg/kg po) to a normal volunteer. At this dose level the plasma concentration can be monitored for at least 5 days for the *S*-isomer or 6 days for the *R*-isomer. The more rapid elimination of the *S*-isomer confirms the findings using the stereospecific MS methods (7, 8) or following the administration of the separate enantiomers (2).

REFERENCES

(1) A. Breckenridge, M. Orme, H. Wesseling, R. J. Lewis, and R. Gibbons, *Clin. Pharmacol. Ther.*, **15**, 424 (1974).
(2) R. A. O'Reilly, *Clin. Pharmacol. Ther.*, **16**, 384 (1974).
(3) J. Koch-Weser, *Eur. J. Clin. Pharmacol.*, **9**, 1 (1975).
(4) R. J. Lewis, W. F. Trager, K. K. Chan, A. Breckenridge, M. Orme, M. Rowland, and W. Schary, *J. Clin. Invest.*, **53**, 1607 (1974).
(5) R. A. O'Reilly, *N. Engl. J. Med.*, **295**, 354 (1976).
(6) R. A. O'Reilly, *N. Engl. J. Med.*, **302**, 33 (1980).
(7) W. N. Howald, E. D. Busch, W. F. Trager, R. A. O'Reilly, and C. H. Motely. *Biomed. Mass Spectrom.*, **7**, 35 (1980).
(8) C. Hignite, J. Vetrecht, C. Tschanz, and D. Arzanoff, *Clin. Pharmacol. Ther.*, **28**, 99 (1980).
(9) C. E. Cooke, N. A. Ballentine, T. B. Seltzman, and C. R. Tallent, *J. Pharmacol. Exp. Ther.*, **210**, 391 (1979).
(10) P. E. Hare and E. Gil-av, *Science*, **204**, 1226 (1979).
(11) C. Gilon, R. Leshman, and E. Goushka, *Anal. Chem.*, **52**, 1206 (1980).
(12) Y. Ta'uhi, N. Miller, and B. L. Karger, *J. Chromatogr.*, **205**, 325 (1981).
(13) B. Silben and S. Riegelman, *J. Pharmacol. Exp. Ther.*, **215**, 643 (1980).
(14) R. W. Souter, *J. Chromatogr.*, **221**, 109 (1980).
(15) K. Imai, S. Mammo, and T. Ohtaki, *Tetrahedron Lett.* **15**, 1211 (1976).
(16) V. du Vigneaud and C. Meyer, *J. Biol. Chem.* **98**, 295 (1932).
(17) B. L. Karger, R. L. Stern, E. Keane, B. Halpern, and J. W. Westley, *Anal. Chem.*, **39**, 228 (1967).
(18) G. Helmchen and G. Nill, *Angew. Chem. Int. Ed. Engl.*, **18**, 65 (1979).
(19) C. G. Scott, M. J. Petrin, and R. McCorkle, *J. Chromatogr.*, **125**, 157 (1976).
(20) H. C. Rose, R. L. Stern, and B. L. Karger, *Anal. Chem.*, **38**, 469 (1966).
(21) G. Halmchen, G. Nill, D. Flockenzi, W. Schükle, and M. S. K. Youssef, *Angew. Chem. Int. Ed. Engl.*, **18**, 62, (1979).
(22) M. Goodman and L. Levine, *J. Am. Chem. Soc.*, **86**, 2918 (1964).
(23) M. Goodman and W. J. McGahren, *Tetrahedron*, **23**, 2031 (1967).
(24) F. Kurzer and K. Douraghi-Zadeh, *Chem. Rev.*, **67**, 107 (1967).
(25) Y. S. Klansner and M. Bodansky, *Synthesis*, **453** (1972).
(26) W. P. Jencks and J. Canivola, *J. Biol. Chem.*, **234**, 1272 (1959).
(27) L. Aarons, *Analyst*, **106**, 1249 (1981).

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Stability-Indicating Assay for Chlorthalidone Formulation: Evaluation of the USP Analysis and a High-Performance Liquid Chromatographic Analysis

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Abstract ☐ An investigation of the USP assay of chlorthalidone tablets showed that variable degradation of chlorthalidone occurred during assay preparation. The degradation products were isolated and identified. A stability-indicating high-performance liquid chromatographic (HPLC) assay which separates the degradation products from chlorthalidone was developed and used to examine the present USP preparation. The HPLC assay is suggested as an alternate.

Keyphrases ☐ Chlorthalidone—high-performance liquid chromatography, stability-indicating assay, comparison with the USP analysis ☐ Degradation products—of chlorthalidone, high-performance liquid chromatographic determination, comparison with the USP analysis ☐ USP analysis—for chlorthalidone and degradation products, comparison to a high-performance liquid chromatographic assay

Chlorthalidone (I) is a monosulfamyl diuretic used in the treatment of hypertension. The official analysis of chlorthalidone tablets, as prescribed in the United States Pharmacopeia, is a spectrophotometric assay (1). Chlorthalidone has been quantitated in various media using a variety of methodologies (2–8). The major techniques in

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