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High-performance liquid chromatographic determination and identification of acyl migration and photodegradation products of furosemide 1-O-acyl glucuronide

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Abstract

Stability of furosemide glucuronide, the major metabolite of furosemide, was studied in order to accurately assess the glucuronidation of furosemide. Furosemide glucuronide was purified by high-performance liquid chromatography, and the mass spectrum of furosemide glucuronide showed the molecular ion peaks $[M-H]^-$ at 505 and 507 (m/z). Furosemide glucuronide was photodegraded to the compound, which was shown more hydrophilic than furosemide glucuronide by high-performance liquid chromatography assay. The photodegradation product of furosemide glucuronide was hydrolyzed to one of the photodegradation products of furosemide by β-glucuronidase, indicating that the photodegradation product of furosemide glucuronide possessed a glucuronic acid moiety. Furthermore, the mass spectrum of the photodegradation product of furosemide glucuronide exhibited molecular ion peaks $[M-H]^-$ at 487 and $[M-2H+2Na]^-$ at 509, indicating the chlorine displacement of furosemide glucuronide by a hydroxyl group. Furosemide glucuronide was unstable in an aqueous solution (pH=7.4), and presumed acyl migration isomers of furosemide glucuronide (furosemide glucuronide-isomers) were detected by high-performance liquid chromatography equipped with photodiode array UV detector. The UV spectra of seven furosemide glucuronide-isomers were closely similar to that of furosemide glucuronide but not furosemide. Exposing a mixture of furosemide glucuronide and furosemide glucuronide-isomers to light resulted in the production of new compounds. UV spectra of photodegradation products of furosemide glucuronide-isomers were closely similar to those of photodegradation product of furosemide glucuronide. These results suggested that furosemide glucuronide-isomers were also photodegraded, resulting in the displacement of chlorine by a hydroxyl group as in furosemide glucuronide. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Photodegradation; Acyl migration; Furosemide

1. Introduction

Furosemide (F), which is widely used as a potent diuretic agent, is metabolized to glucuronic acid conjugate, furosemide 1-O-acyl glucuronide (Fgnd), in human [1–3]. Rachmel reported that Fgnd was hydrolyzed to F or isomerized to five isomers by acyl migration [4]. F has been also reported to be

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hydrolyzed to 4-chloro-5-sulfamoylanthranilic acid (CSA) [5] or to be photodegraded to CSA and several other compounds [6]. Furthermore, preliminary study suggested that Fgnd was so unstable under light (Sekikawa et al., unpublished data). These characteristics of Fgnd and F cause misunderstanding the pharmacokinetics of F and Fgnd. In order to accurately evaluate glucuronidation of F, it is required to clarify the stability of Fgnd. Since the preliminary study was done with partially purified Fgnd, in this report we purified Fgnd, and identified acyl migration isomers and photodegradation products of Fgnd by UV spectrometry and/or mass spectrometry analysis.

2. Experimental

2.1. Chemicals

Furosemide and β-glucuronidase (type L-II) were obtained from Sigma (St. Louis, MO, USA). CSA was obtained from U.S. Pharmacopoeia (Rockville, MD, USA). Acetonitrile and methanol (HPLC grade) were obtained from J.T. Baker (Phillipsburg, NJ, USA). Fgnd was obtained by the method described below [7]. All other chemicals were of reagent grade.

2.2. Isolation and identification of Fgnd

Isolation and identification of Fgnd was performed by the method reported previously [7]. Briefly, Fgnd was extracted with ethyl acetate from the urine, which was collected for 8 h following the oral administration of furosemide (40 mg) according to the method reported previously [7]. Two hundreds microliters of crude Fgnd dissolved in the mobile phase was applied to a semipreparative HPLC column (Alltech C18, 10 mm I.D.×25 cm length, 10 μm particle size) with mobile phase (30% acetonitrile, 0.1 *M* ammonium acetate, pH=3.0) at a flow-rate of 3 ml/min. The fraction of Fgnd, of which retention time was around 19.5 min, was collected. Finally, purified Fgnd was obtained by freeze-drying. Mass spectrum of purified Fgnd was obtained with the negative ion mode of liquid secondary ion mass spectrometry (LSIMS), showing molecular ion peaks, $[M-H]^-$ at 505 and 507 (m/z) with peak ratio of 3:1 representing one chlorine in this molecule (data not shown). For checking Fgnd in each step, analytical HPLC was performed with an ECONOSIL C18 analytical column (4.6 mm I.D.× 25 cm length, 5 μm particle size, Alltech/Applied Science), a Kratos Spectroflow 783 UV detector (Kratos Analytical, 170 Williams, Drive Ramsey, NJ), a Perkin–Elmer 204-S fluorescence spectrophotometer (Perkin–Elmer Co. Norwalk, Connecticut) and the HPLC pump (1.5 ml/min flow-rate). The mobile phase was 30% acetonitrile and 0.05% phosphoric acid in purified water (pH=3.0). The hydrolysis of Fgnd by β-glucuronidase was performed by incubating the purified Fgnd in 0.1 *M* acetate buffer (pH=5.0) containing β-glucuronidase (1000 unit/ml) for 4 h.

2.3. Photodegradation experiments

Sample solution of Fgnd, Fgnd-isomer or F in glass tubes (Borosilicate Disposable Culture Tubes, 13 mm I.D.×100 mm, Fisher Scientific Co., Pittsburgh, PA) were placed under sunlight through a glass of window for 30 min (Fgnd) and 8 h (F). UV spectra of compounds were determined by the HPLC system, which consisted of an HP 1040 photodiode array UV detector (Hewlett Packard, Federal Republic of Germany) and an Altex Ultrasphere-ODS (5 μm, 4.6 mm I.D.×250 mm length, Beckman Instruments Inc., Berkeley, CA) connected to a Rainin Rabbit HP/HPX Drive Module pump controlled by Mac RABBIT (Rainin Instrument Co., Inc. Mack Road, Woburn, MA). The rest of assay conditions were described in Figure Legends.

The photodegradation product was collected from a solution of Fgnd in phosphate buffer (pH=3.5) exposed to sunlight for 15 min, using the analytical column and a mobile phase consisting of 20% acetonitrile in 0.1 *M* ammonium acetate buffer (pH=3.5). The product, of which the retention time was previously determined, was collected while the UV lamp was turned off. The collected sample was freeze-dried. Mass spectra were obtained by the method described above. The photodegradation product of Fgnd was incubated in 0.1 *M* sodium acetate buffer (pH=5.0) containing β-glucuronidase (1000 units/ml) for 6 h at 37°C.

Due to the rapid photodegradation of Fgnd, hand-

ling Fgnd and F were done in a dark room or under fluorescent lamps covered with translucent plastic frames at night. For the automatic HPLC assays, glass inserts were covered with aluminum foil to protect the specimens from light.

2.4. Acyl migration experiment

Fgnd in a 0.15 *M* potassium phosphate buffer (pH=7.4) were incubated at 37°C according to the method of the previous study [8], and were immediately analyzed with the HPLC system equipped with photodiode array UV detector, which was described above. Mobile phases used in the gradient method were: Solvent A; 17.6% acetonitrile, 3.6% methanol, 0.05% phosphoric acid in water at pH=3.5 and Solvent B; 100% acetonitrile. The flow-rate was 1.3 ml/min. The gradient program was set as follows. During the time 0 to 34.9 min the mobile phase was 100% Solvent A, and from 35.0 to 49.9 min the mobile phase consisted of 70% Solvent A and 30% Solvent B. Then, 100% Solvent A was flowed during the time 50.0 to 60.0 min.

3. Results and discussion

3.1. HPLC chromatogram of photodegradation product of Fgnd

Fig. 1 depicts a chromatogram of Fgnd exposed to sunlight for 5 min. A compound with a retention time of 5 min was observed in addition to that of Fgnd (retention time, 19.5 min). Overlaid on the chromatogram in Fig. 1a is a chromatogram of pure CSA measured under the same conditions.

3.2. UV spectrum of photodegradation product of Fgnd

Fig. 1 shows the UV spectra of CSA, Fgnd and a photodegradation product of Fgnd. The UV spectrum of a photodegradation product of Fgnd was completely different from those of Fgnd, F and CSA (the hydrolysis product of F [5]). However, the retention time of CSA was close to that of a photodegradation product of Fgnd (Fig. 1), indicating a possibility for misidentification of CSA in biological samples.

3.3. Mass spectrum of photodegradation product of Fgnd

A mass spectrum of the photodegradation product of Fgnd showed molecular ion peaks, $[M-H]^-$ at 487 and $[M-2H+2Na]^-$ at 509, indicating that the chlorine was substituted by a hydroxyl group (Scheme 1). That is, the molecular ion peak $[M-H]^-$ at 487 was less than that of Fgnd by 18 or 20 mass unit.

3.4. β-Glucuronidase treatment of the photodegradation product of Fgnd

After β-glucuronidase treatment of the photodegradation product of Fgnd, the peak of the photodegradation product of Fgnd (retention time, 5 min) on the HPLC chromatogram disappeared, and another compound was detected around 11 min (data not shown). This suggests that the photodegradation product of Fgnd has a glucuronic acid moiety (Scheme 1). The UV spectrum of β-glucuronidase-treated photodegradation product of Fgnd was different from that of Fgnd or photodegradation product of Fgnd (Fig. 2). Furthermore, the retention time (11 min) of HPLC and UV spectrum (Fig. 2) of the β-glucuronidase-treated photodegradation product of Fgnd were identical to that of one of the F photodegradation products (chlorine displaced furosemide) (Fig. 2), of which mass spectrum showed molecular ion peak $[M-H]^-$ at 311. This supports the proposed structure of the photodegradation product of Fgnd (Scheme 1).

The photodegradation of F in oxygen-free methanol has been reported to result in the formation of both CSA and F derivative, in which chlorine was substituted by a hydrogen or a methoxy group [6]. The substitution reaction induced by light has been also reported for hydrochlorothiazide [9], in which hydrochlorothiazide underwent substitution of chlorine by a hydroxyl group in an aqueous solution, or by a methoxy group or a hydrogen in a methanol solution. On the other hand, the HPLC chromatogram of Fgnd in methanol exposed to sunlight showed a single peak, which was identical to that of Fgnd itself (data not shown). The mass spectrum of Fgnd in methanol exposed to sunlight also showed molecular ion peaks $[M-H]^-$ at 505 and 507 (m/z),

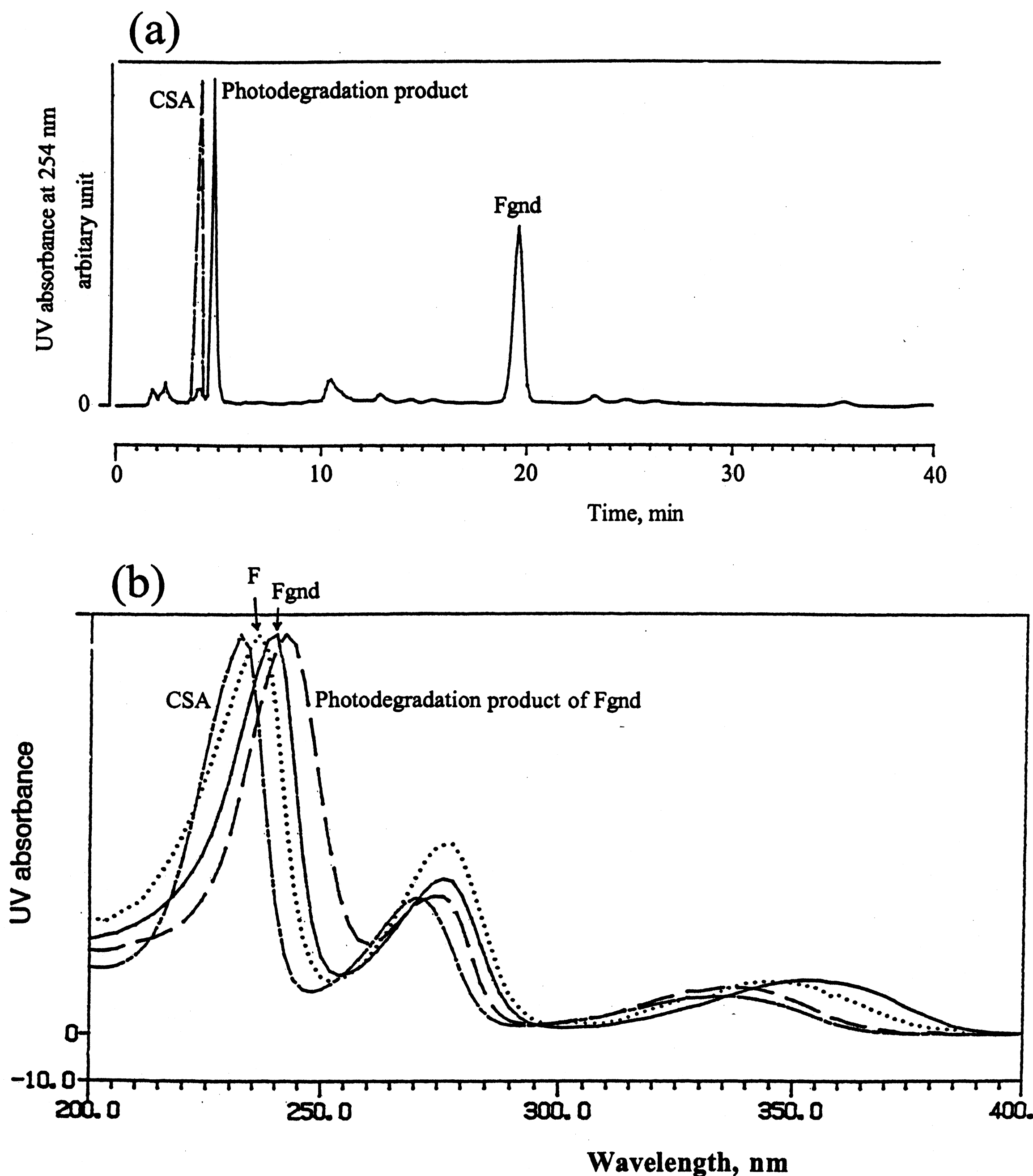
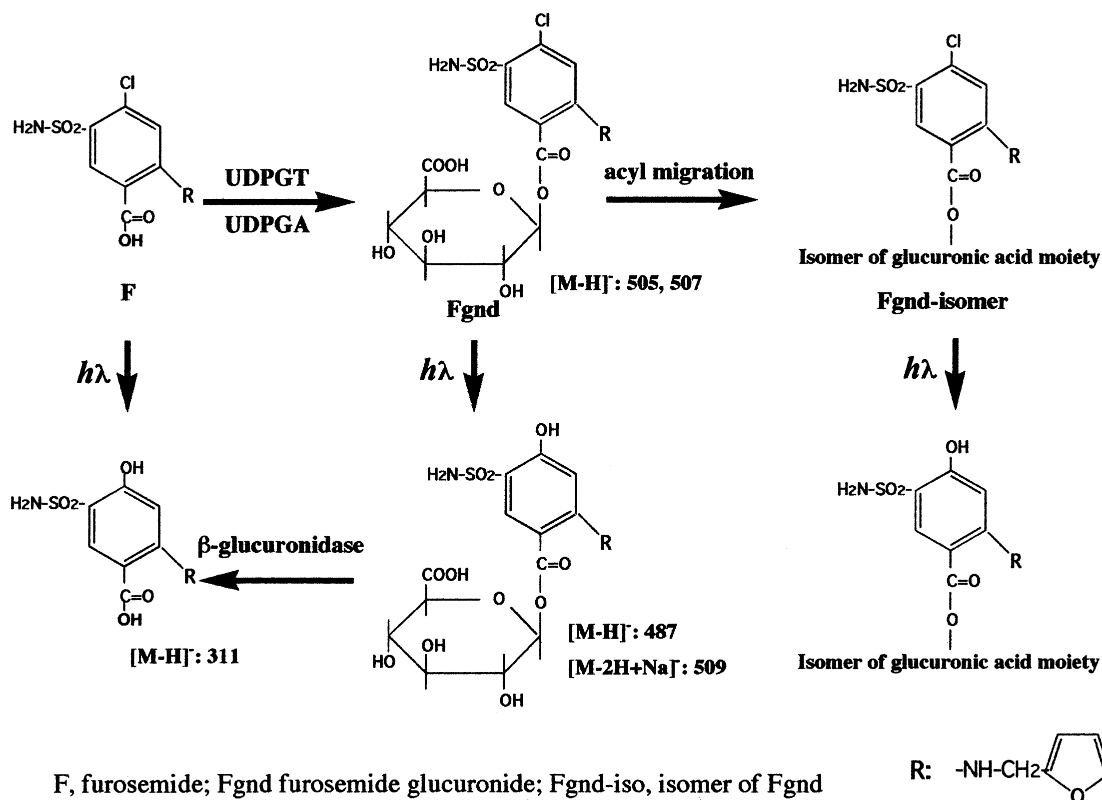


Fig. 1. HPLC chromatogram (a) and UV spectra (b) of photodegradation product of Fgnd. (a) Chromatogram of photodegradation products of Fgnd. The chromatogram of pure CSA (4 min retention time) was overlaid. The purified Fgnd was exposed to sunlight for 5 min before HPLC assay. Mobile phase: 20% acetonitrile, 0.05% phosphoric acid, pH=3.0. flow-rate: 1.5 ml/min. Analytical column was used, see text. (b) UV spectra of F, Fgnd, CSA and photodegradation product of Fgnd were shown. The condition of the HPLC system was the same as in (a).



Scheme 1. Scheme showing proposed pathways of photodegradation and acyl migration of Fgnd and its isomers.

which was identical to that of Fgnd itself. Moore and coworkers [6,10] reported that oxygen in methanol strongly inhibited the photodegradation of F, and that the suppressed photodegradation of F in methanol was attributed to oxygen quenching of the triplet state of the molecule induced by irradiation. Therefore, it is concluded that the photodegradation of Fgnd was prevented by oxygen dissolved in methanol. Therefore, methanol must be a good solvent for Fgnd sample handling.

3.5. HPLC chromatograms and UV spectra of presumed acyl migration products

Fig. 3 shows the chromatograms of the samples in the HPLC assay, which were obtained after incubation of Fgnd in 0.15 *M* potassium phosphate buffer (pH=7.4) at 37°C for 1.5 and 36 h, respectively. A primary peak of Fgnd was observed in the sample after incubation of Fgnd for 1.5 h (Fig. 3), whereas several peaks other than those of Fgnd and F were observed in the sample after incubation of Fgnd for 36 h (Fig. 3). The formation of these peaks were not observed in the sample after incubation of Fgnd solution at pH=3.5 (data not shown). The profile of this chromatogram obtained by UV detector (Fig. 3) was similar to that obtained by the preliminary experiment using crude Fgnd and fluorescence detector [8]. The UV spectra of the peaks I–VIII in Fig. 3, are shown in Fig. 4. The UV spectra of F, Fgnd (peak number VII) and one of new compounds (peak number VIII) are shown in Fig. 4. The UV spectrum (maximum wavelength of absorption, λmax; 238 nm) of the compound of peak VIII differed from that (λmax; 236 nm) of F, but was closely similar to that (λmax; 240 nm) of Fgnd itself. Furthermore, UV spectra (λmax of all spectra were 238 nm) of seven new peaks were closely similar to each other (Fig.

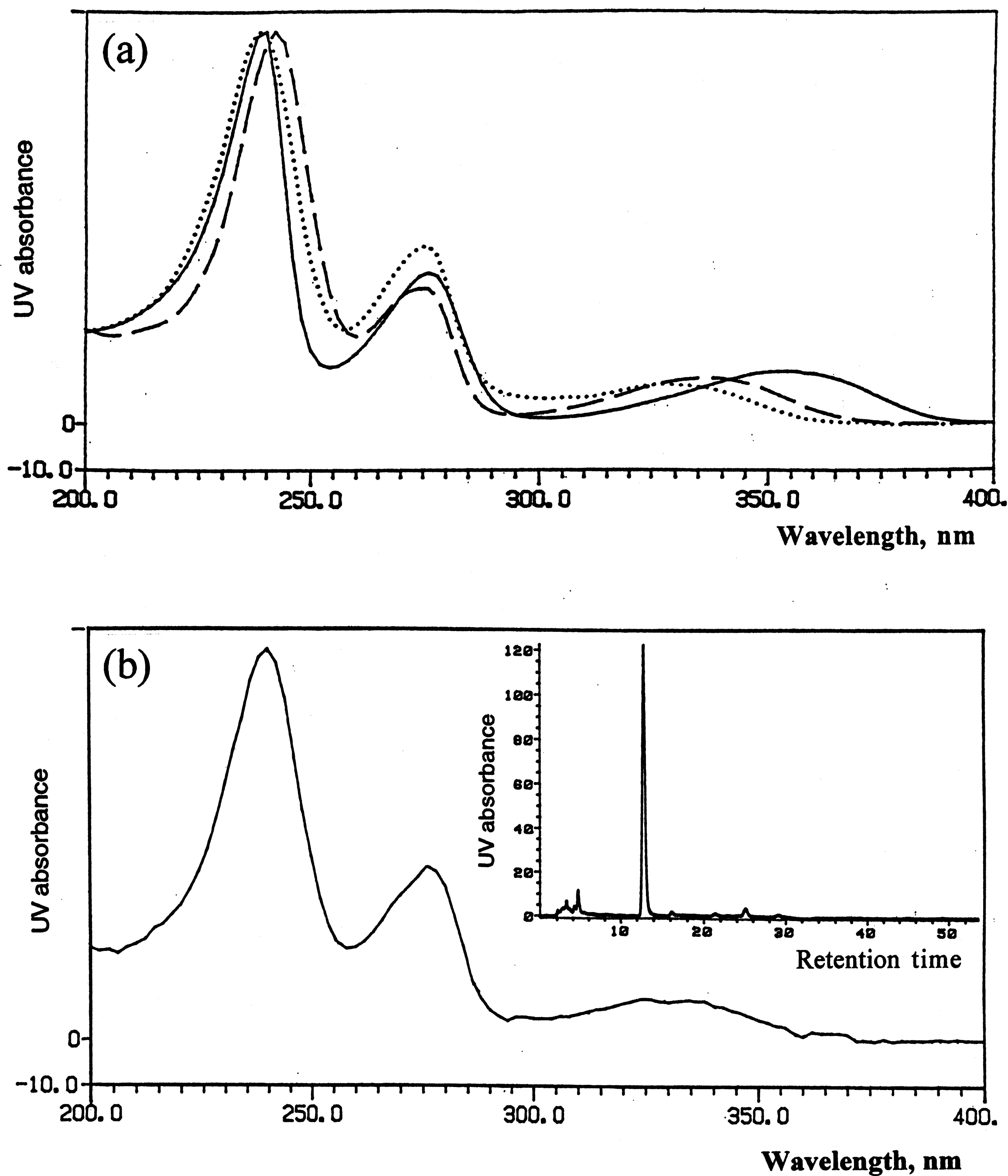


Fig. 2. Comparison of UV spectra of Fgnd and relate compounds with Fgnd. (a) UV spectra of Fgnd, the compound formed by β-glucuronidase treatment of photodegradation product of Fgnd, and the Fgnd photodegradation product. (...........) β-glucuronidase-treated photodegradation product of Fgnd, (- - -) photodegradation product of Fgnd, (———) Fgnd. (b) HPLC chromatogram and UV spectrum of one of photodegradation products of F. Mobile phase: 20% acetonitrile, 0.05% phosphoric acid, pH=3.0. Flow-rate: 1.5 ml/min. Analytical column was used, see text.

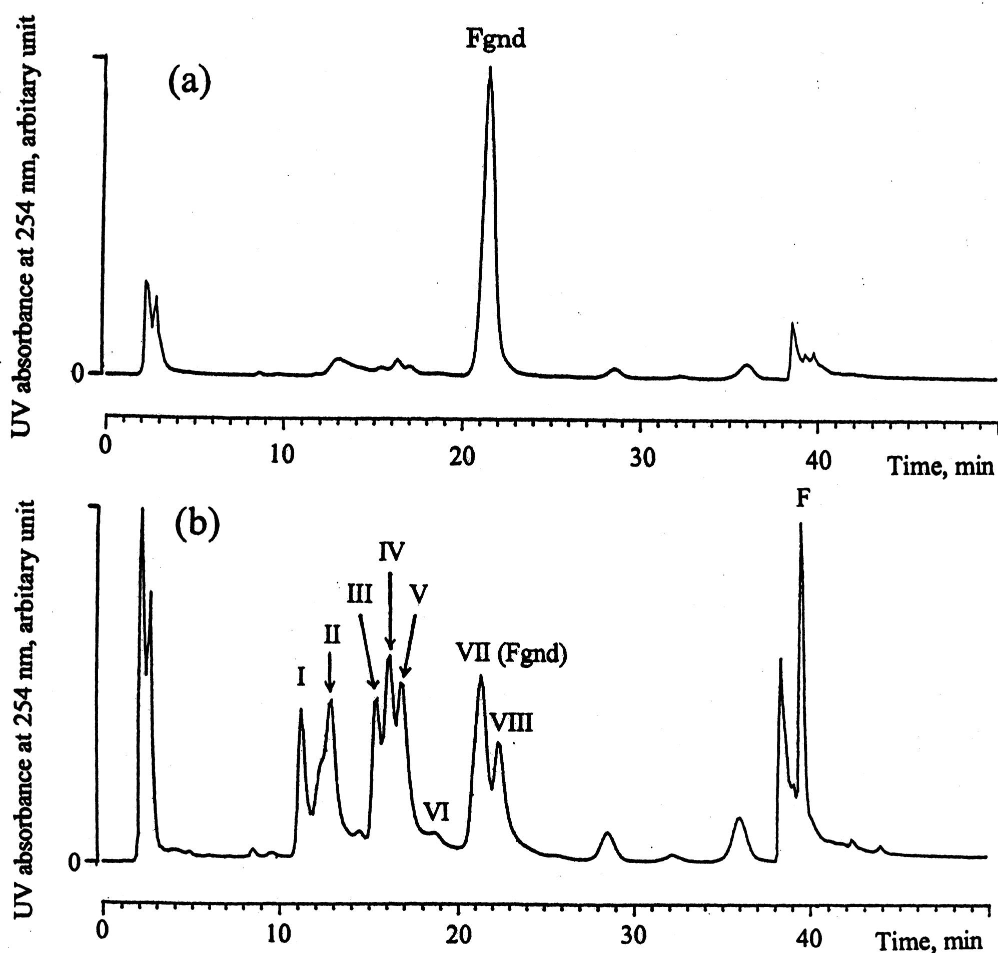


Fig. 3. HPLC chromatograms showing instability of Fgnd in pH=7.4 buffer at 37°C. Chromatograms were obtained by HPLC–UV (254 nm). Incubation time: 1.5 h (a), 36 h (b). Fgnd, furosemide glucuronide; F, furosemide.

4). Previous study [8] showed that only Fgnd was hydrolyzed by β-glucuronidase, and the compounds of all peaks detected by fluorescence detector were hydrolyzed with 0.5 *M* sodium hydroxide, resulting in an increase of F. Therefore, these results indicate that the degradation products of Fgnd were acyl migration isomers of the glucuronic acid moiety of Fgnd (Scheme 1). Our results support the report of Rachmel et al. [4], where 6 isomers of Fgnd were detected by fluorescence detector.

Several researchers have reported the presence of more than four isomers of the 1-β-D-glucuronides of drugs (glucuronides of zomepirac [11,12], isoxepac [13] and valproic acid [14]). Dickinson et al. [15] reported the intramolecular rearrangement of valproic acid glucuronide, speculating on the presence of isomers containing 2, 3, and 4-O-acyl-α and β-glucopyranose and the presence of furanose and lactose derivatives formed from the open-chain intermediates. Seven isomers of Fgnd observed in this study were considered to account for α- and β-forms of 2, 3, and 4-O-acyl glucuronide isomers and α-form of 1-O-acyl glucuronide which were formed by acyl migration and intermediation with open-chain in glucuronic acid moiety [15].

3.6. Photodegradation of Fgnd-isomers

Fig. 5 shows the HPLC chromatogram of Fgnd-isomers exposed to sunlight. This indicates the

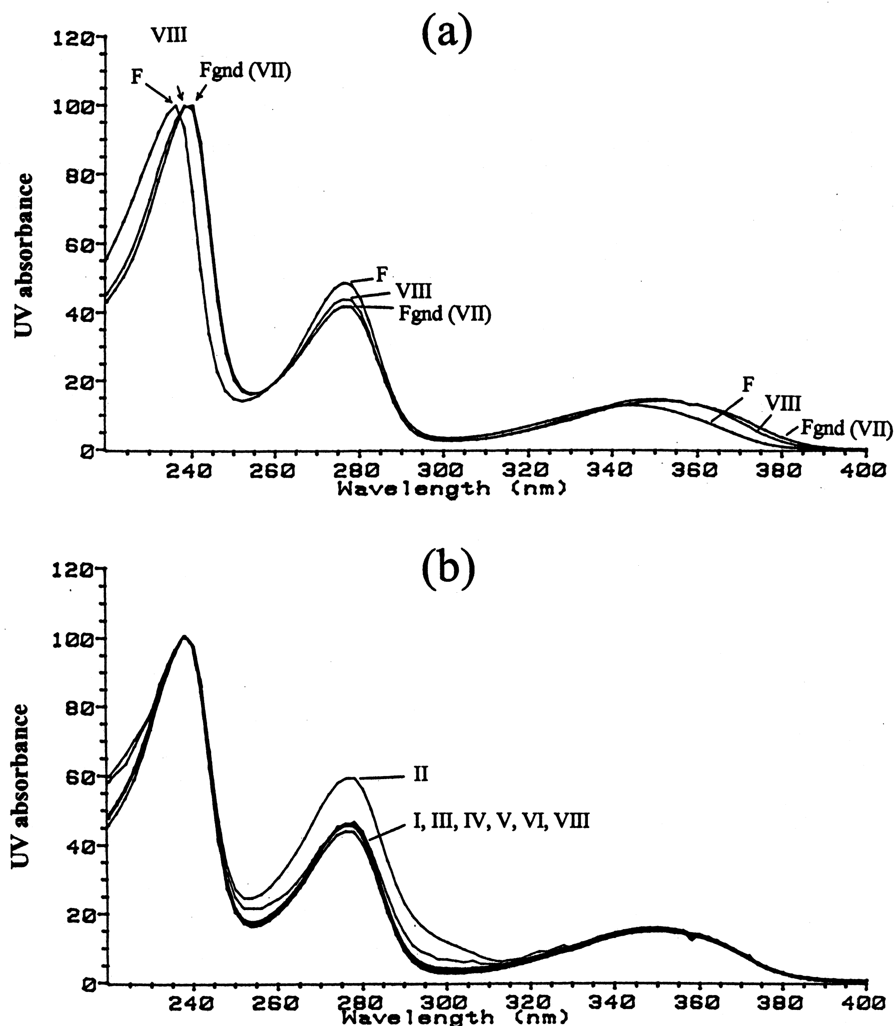


Fig. 4. UV spectra of F, Fgnd and products from Fgnd. (a) UV spectra of F, Fgnd and a new compound formed from Fgnd in pH=7.4 buffer at 37°C. A new compound is of peak ≠VIII in Fig. 3b. (b) UV spectra of new peaks (peak number I–VIII excluding number VII) shown in Fig. 3b.

exposure of Fgnd-isomers to sunlight resulted in degradation of Fgnd-isomers and in formation of several compounds. The UV spectrum of the compound, which was eluted earlier than that of the Fgnd degradation product, was nearly identical to that of the Fgnd degradation product (Fig. 5). Since Fgnd photodegradation products were highly hydrophilic, these were not clearly separated by the HPLC. When the mobile phase was more hydrophilic, three peaks were detected around 10 min. UV spectra of three compounds eluted around 10 min were nearly identical to that of the photodegradation product of Fgnd shown in Fig. 3 (data not shown). These results strongly suggest that photodegradation of Fgnd-isomers also occurred (Scheme 1). Bundgaard et al. reported that alkyl esters of F in an aqueous solution was unstable under light to form the compound, of which chlorine was displaced with hydroxyl group [16]. Therefore, it is concluded that the esterification of carboxyl group of F with glucuronic acid and its isomers as well as alkyl group enhanced the photo-instability of furosemide.

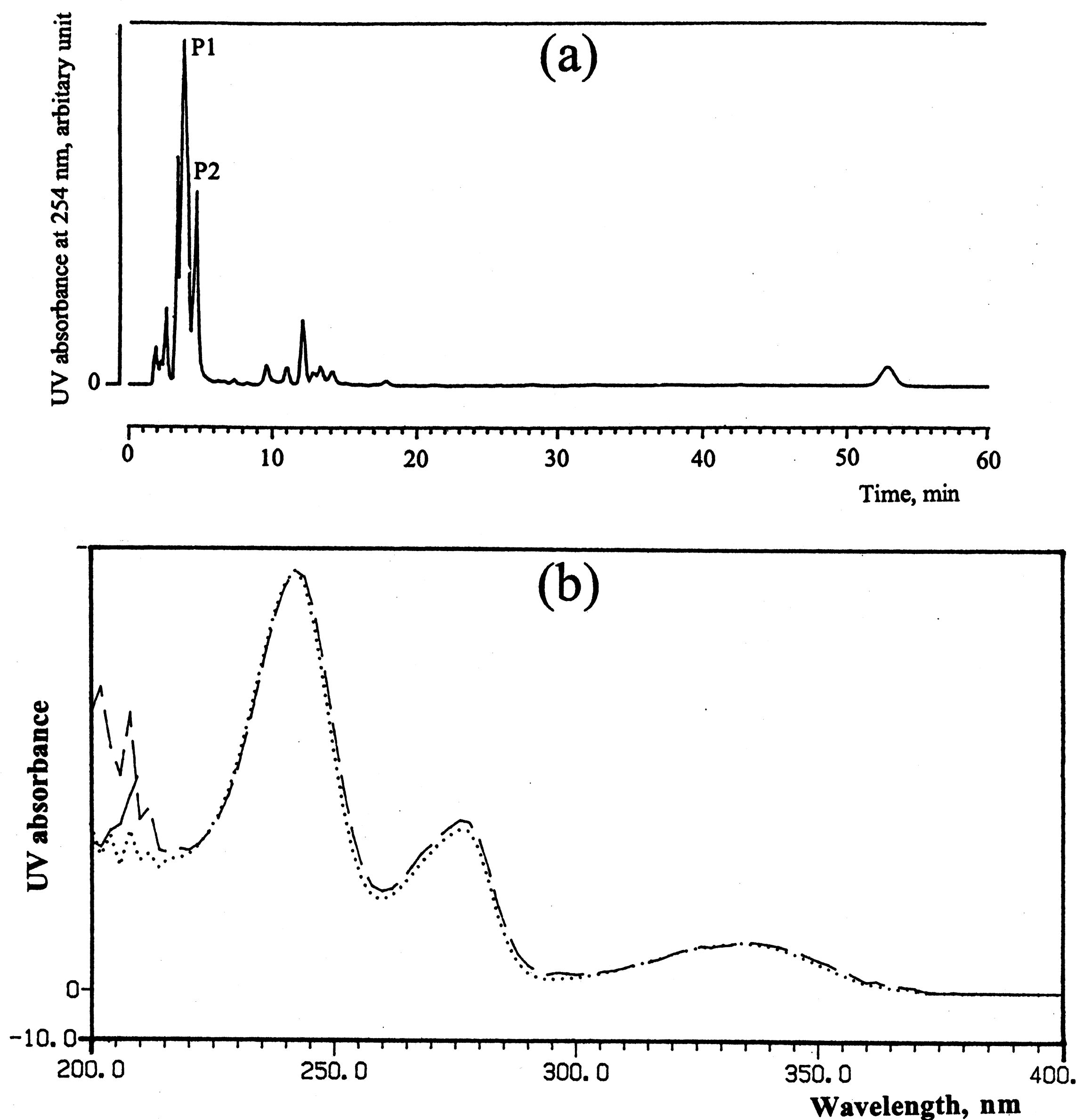


Fig. 5. HPLC chromatogram (a) and UV spectra (b) of photodegradation products of Fgnd and Fgnd-isomers. UV spectra: photodegradation products of Fgnd-isomers (.........., P1 shown in (a)) and Fgnd (- - -, P2 shown in (a)). Mobile phase: 20% acetonitrile, 0.05% phosphoric acid, pH=3.0. Flow-rate: 1.5 ml/min. Analytical column was used, see text.

In conclusion, this study proposed the following pathways of Fgnd degradation. Fgnd is photodegraded to chlorine-displaced compound with an hydroxyl group. The photodegradation product of Fgnd was more hydrophilic than Fgnd by HPLC assay. Fgnd in pH=7.4 solution is unstable, forming at least seven isomers by acyl migration, but not in the lower pH solution. UV spectra of Fgnd and Fgnd-isomers are closely similar to each other. Fgnd-isomers are also photodegraded to the compound, of which UV spectrum is closely similar to that of phlodegradation product of Fgnd. Therefore, in order to accurately

determine Fgnd, lowering the pH of Fgnd solution and light-protection are required in handling of Fgnd sample.

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References

[1] D.E. Smith, E.T. Lin, L.Z. Benet, Drug Metab. Dispos. 8 (1980) 337.
[2] J. Perez, D.S. Sitar, R.I. Ogilvie, Drug Metab. Dispos. 7 (1979) 383.
[3] A.L.M. Kerremans, Y. Tan, C.A.M. Van Ginneken, F.W.J. Gribnau, J. Chromatogr. 229 (1982) 129.
[4] A. Rachmel, G.A. Hazelton, A.L. Yergey, D.J. Liberato, Drug. Metab. Dispos. 13 (1985) 705.
[5] J.E. Cruz, D.D. Maness, G.J. Yakatan, Int. J. Pharm. 2 (1979) 275.
[6] D.E. Moore, V. Sithipitaks, J. Pharm. Pharmacol. 35 (1983) 489.
[7] T. Mizuma, L.Z. Benet, E.T. Lin, Prep. Biochem. Biotechnol. 28 (1998) 37.
[8] H. Sekikawa, N. Yagi, E.T. Lin, L.Z. Benet, Biol. Pharm. Bull. 18 (1995) 134.
[9] S.R. Tamat, D.E. Moore, J. Pharm. Sci. 72 (1983) 180.
[10] D.E. Moore, S.R. Tamat, J. Pharm. Pharmacol. 32 (1980) 172.
[11] J. Hasegawa, P.C. Smith, L.Z. Benet, Drug Metab. Dispos. 10 (1982) 469.
[12] P.C. Smith, L.Z. Benet, Drug Metab. Dispos. 14 (1986) 503.
[13] H.P.A. Illing, I.D. Wilson, Biochem. Pharmacol. 30 (1981) 3381.
[14] R.G. Dickinson, W.D. Hooper, M.J. Eadie, Drug Metab. Dispos. 12 (1984) 247.
[15] R.G. Dickinson, R.M. Kluck, M.J. Eadie, W.D. Hooper, J. Pharmacol. Exp. Ther. 233 (1985) 214–221.
[16] H. Bundgaard, T. Norgaard, N.M. Nielsen, Int. J. Pharm. 42 (1988) 217.