Department of Pharmacy, Free University of Berlin, Germany

# Photoallergenic potential testing by online irradiation and HPLC

S. SCHRÖDER, P. SURMANN

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*Prof. Dr. Peter Surmann, University of Berlin, Department of Pharmacy, Königin-Luise-Str. 2+4, 14195 Berlin, Germany*
*psurmann@zedat.fu-berlin.de*

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A simple screening method was developed for testing the photoallergenic potential of drugs. The photolysis of the drugs under UV-A irradiation was investigated in aqueous buffer solution alone and in combination with a model dipeptide. As an useful tool for this, high performance liquid chromatography (HPLC) based on an automated column-switching system with aqueous online UV-A irradiation was used. For testing the system, hydrochlorothiazide (HC) with well-known photoallergenic properties was used as a drug and alanyltryptophane (ALATRP) as a model for proteins. Promethazine (PMZ) as a second drug was investigated by the developed screening system. The screening was performed at pH 3 and pH 7. Both cleavage of the peptide binding and formation of new products could indicate a photoallergenic potential.

## 1. Introduction

Drug induced photoallergy is a consequence of the exposure of treated patients to sunlight (Horio et al. 1994; Moore 2002). The mechanism of photoallergic reactions involves covalent drug-protein photobinding (heptanization) leading to the formation of a photoantigen. The photosensitised modification of the protein may produce extensive structural changes associated with loss of biological function. The reaction of drugs with large proteins is difficult to investigate, therefore in this study the dipeptide alanyltryptophane (ALATRP) was used as a model protein. One reason for using the peptide as model substance is that TRP is one of the three amino acids that have UV chromophores; moreover it has also fluorescence properties. Another reason is the sensitivity of TRP against singulet oxygen. The attack of reactive singulet oxygen results in the damage of TRP (Davies 2004). Either the cleavage of the peptide bond or formation of any reaction products with hydrochlorothiazide (HC) caused by irradiation could be interpreted as an indication of possible change in the structure of proteins and therefore as photoallergy risk.

At presence, photoallergy testing is mainly conducted using guinea pig models. Neumann et al. (2005) evaluated different *in vitro* and *in vivo* methods. Furthermore *in vivo* models like modified LLNA-assay and MEST-assay are under development. An *in vitro* screening method could, however, be a useful alternative.

Hydrochlorothiazid (HC) belongs to the class of thiazided, which are used as diuretics. This drug produced cutaneous reactions, such as vasculitis, erythema multiforme and photosensitivity (Han 2000). Photolytic decomposition of HC was also reported (Swasano et al. 1983). Promethazine (PMZ) belongs to the phenothiazines used as neuroleptics. The phenothiazines, especially promethazine cause photoallergic reaction.

## 2. Investigations, results and discussion

Photodegradation studies of HC and PMZ were carried out for 10, 20, 30, 40, 50 min under UV-A exposure at pH 3 and pH 7; the concentration of the aqueous phosphate buffer solution was 5 mmol/l. After the degradation analysis of HC or PMZ, a mixture of HC or PMZ and ALATRP solution was treated under the same conditions.

The temperature in the photochemical reactor was hold at 35–36 °C. This temperature was chosen to simulate the skin temperature.

Fig. 1 shows four chromatograms of the screening study of HC and ALATRP. Chromatogram A shows the unexposed sample of HC in presence of ALATRP in equimolar concentration, buffered at pH 3. Chromatogram B shows the 50 min irradiated sample of ALATRP and chromatogram C shows the 50 min UV-A irradiation of HC. The peaks P1 and P2 are unidentified photoproducts of HC. The last chromatogram shows the 50 min irradiation of HC in presence of ALATRP. When HC is irradiated in presence of ALATRP four new photoproducts appeared that are not formed upon irradiation of either component alone. The photoproduct at 19.8 min is the peptide TRP, identified by an authentic standard solution of TRP.

The kinetic data of degradation of pH 3 HC solution are shown in Fig. 2. After 50 min of UV-A irradiation the HC area decreased to 15.8%. Fig. 3 shows the kinetic curves of the irradiation of HC in presence of ALATRP.

The photochemical kinetic curve of HC in presence of ALATRP at pH 3 (Fig. 3) was comparable with the irradiation of HC alone at pH 3 (Fig. 2). As shown in Fig. 3, a decrease of the ALATRP curve was observed. The peptide damage induced by UV-A light increased with irradiation time. In addition of HC and UV-A irradiation the ALATRP concentration reached a value of 60.8% after 50 min irradiation. The peak area of the identified TRP simultaneously increased to 21.9% after 50 min irradiation. The decrease of ALATRP in presence of HC and UV-A is significant in comparison with the unirradiated kinetics in Fig. 4 and it is also significant in comparison with the kinetics of irradiated ALATRP alone in Fig. 5.

The ALATRP irradiated alone reaches a concentration of 94% after 50 min irradiation. Without irradiation of HC in presence of ALATRP no effects were observed.

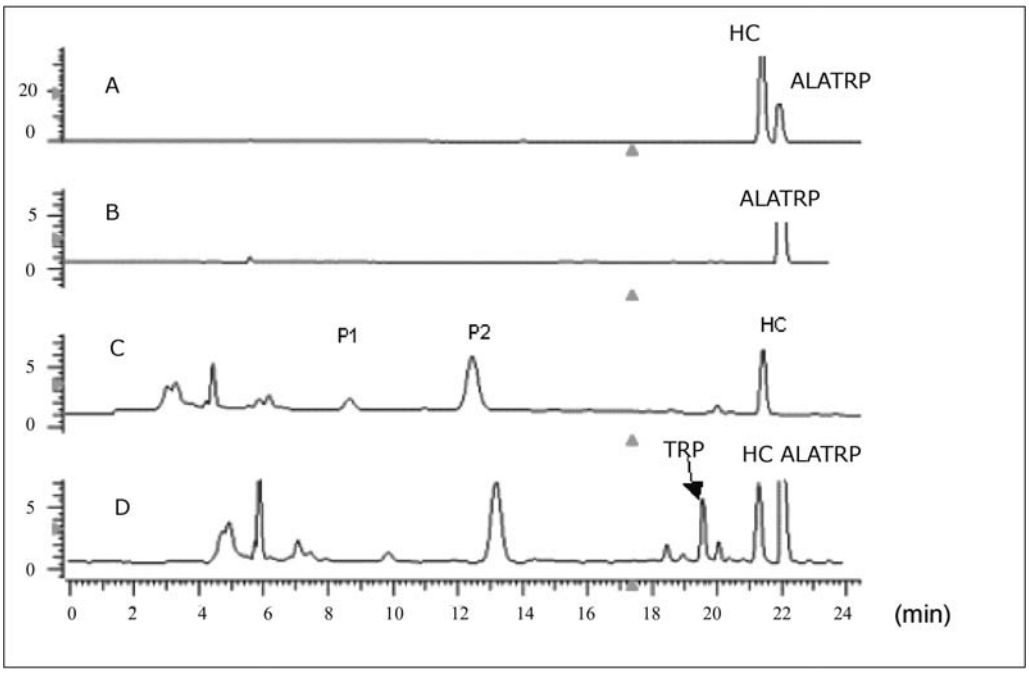


Fig. 1: Chromatograms (A) shows the combined HC and ALATRP solution unexposed; (B) the solution of ALATRP, (C) the solution of HC and (D) the combined solution of HC and ALATRP exposed for 50 min to UV-A at pH 3

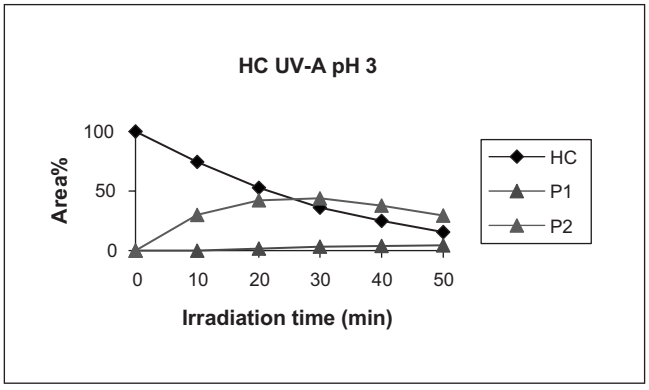


Fig. 2: Photodegradation kinetics of HC at pH 3, exposed to UV-A irradiation

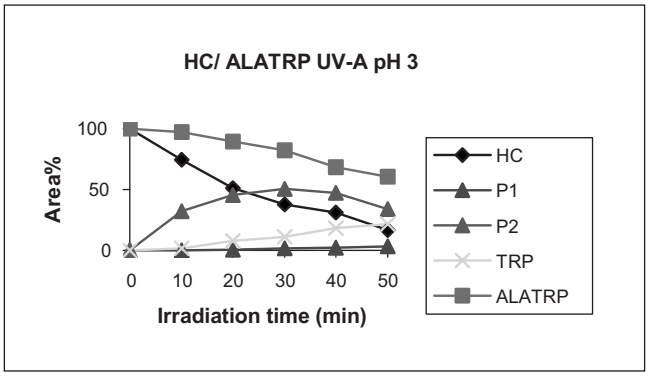


Fig. 3: Photodegradation kinetics of HC and formation of TRP, in presence of ALATRP at pH 3, exposed to UV-A irradiation

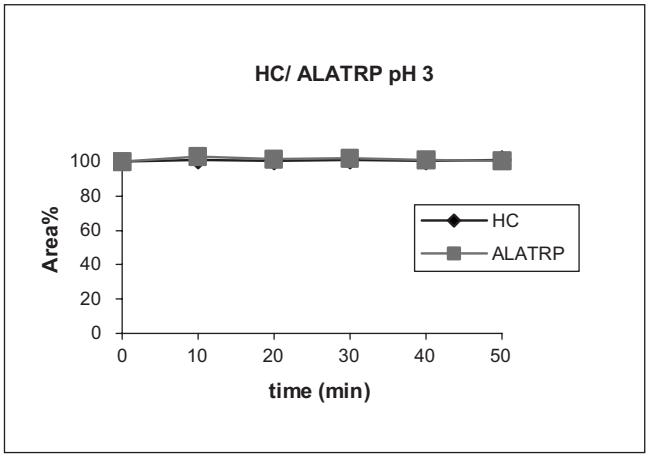


Fig. 4: Kinetics of combined HC and ALATRP solution, at pH 3 unexposed

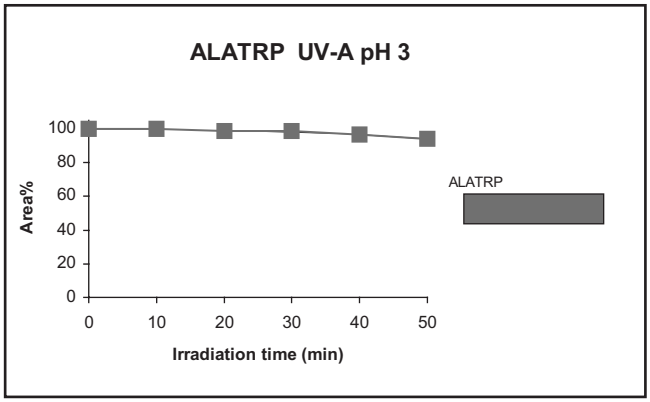


Fig. 5: Photodegradation kinetics of ALATRP at pH 3, exposed to UV-A irradiation

The described investigation of the drug and the dipeptide has been also carried out at pH 7. For the irradiation and separation study a phosphate buffer was used. The results of the pH 7 analysis exhibit no reaction between HC and ALATRP.

Fig. 6 shows four chromatograms of the PMZ/ALATRP experiment. Chromatogram A was desired from the unirradiated probe of the drug and the dipeptide in equimolar concentration. This chromatogram shows the two peaks of the both educts. Chromatogram B shows the 50 min irradiated sample of ALATRP and chromatogram C the 50 min irradiation of PMZ alone. The last chromatogram D is the result of 50 min irradiation of PMZ in presence of ALATRP. The photoproduct which occurs by

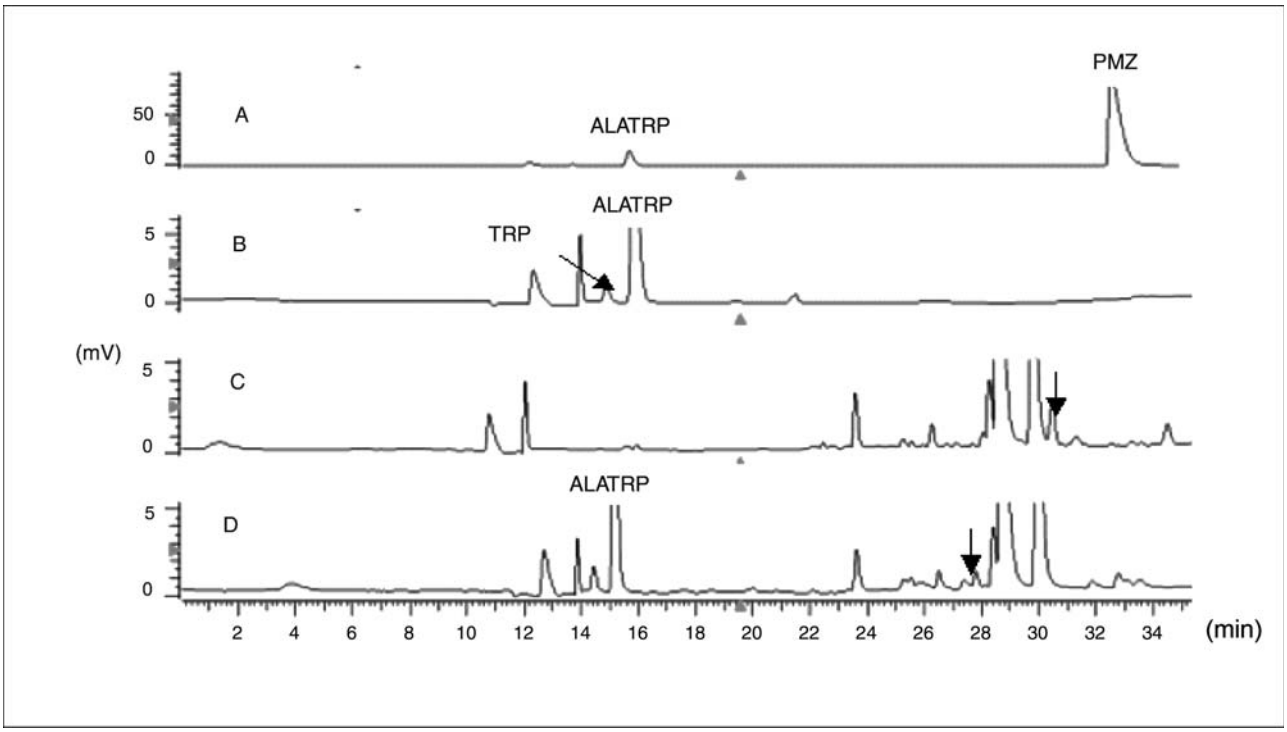


Fig. 6: Chromatograms (A) shows the combined PMZ and ALATRP solution unexposed; (B) the solution of ALATRP, (C) the solution of PMZ and (D) the combined solution of PMZ and ALATRP exposed for 50 min to UV-A at pH 7

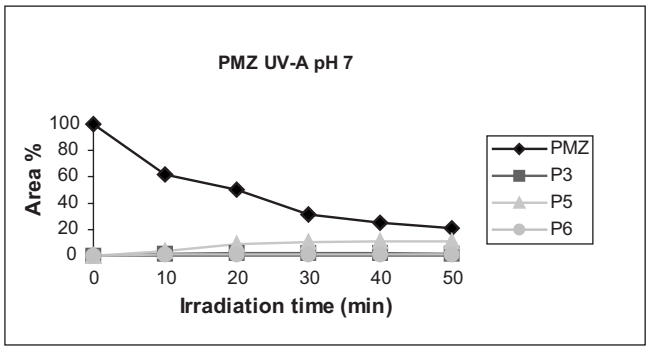


Fig. 7: Photodegradation kinetics of PMZ at pH 7, exposed to UV-A irradiation

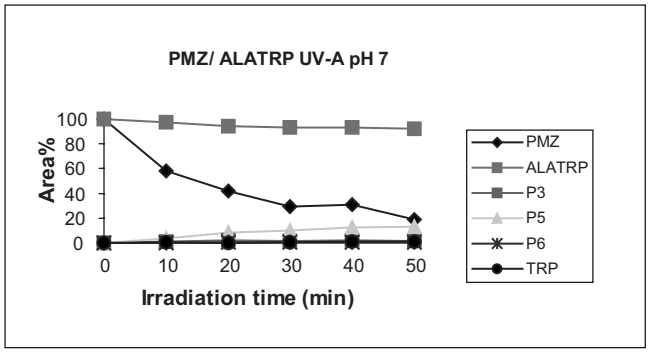


Fig. 8: Photodegradation kinetics of PMZ in presence of ALATRP at pH 7, exposed to UV-A irradiation

the irradiation of the PMZ sample in chromatogram C, marked with an arrow, cannot be identified in the other chromatograms. However, in chromatogram D which was generated after 50 min irradiation of PMZ/ALATRP two new peaks can be seen. These two new peaks marked with an arrow, could not be identified by the separate irradiation of ALATRP or PMZ in the chromatograms B/C. This result indicates some reaction between the drug and the peptide, maybe a new product was formed from the light activated drug and peptide.

Fig. 7 shows the kinetics of irradiated PMZ in absence of ALATRP. After 50 min UV-A irradiation, a decrease of the PMZ peak was observed, the peak area reached approximately 21%.

The results of combined irradiation of PMZ/ALATRP are given in Fig. 8. The diagram presents the kinetic curves of PMZ, ALATRP, the identified photoproduct TRP and three unidentified photoproducts of PMZ.

The decrease of the kinetic curve of ALATRP shown in Fig. 8 is significantly different from the curve of unirradiated ALATRP shown in Fig. 9 and it is also from the curve of the ALATRP irradiated alone shown in Fig. 10.

Both investigated drugs exposed to UV-A-light show photodegradation depending on the pH of the solution. The solved dipeptide ALATRP is resistant to UV-A-light but in the presence of the investigated photoactive drugs reactions occur to the peptide leading to the cleavage of the peptide bond and also to other unidentified products. Because parts of natural proteins could act as allergens, drugs causing the cleavage of the peptide bond have photoallergenic potency. Therefore instead of an animal experiment the proposed method can be a first screening method for testing the photoallergenic potential of a new drug.

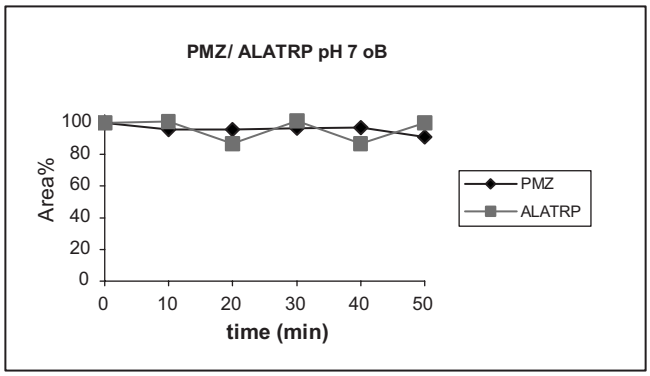


Fig. 9: Kinetikcs of combined PMZ and ALATRP solution, at pH 7 unexposed

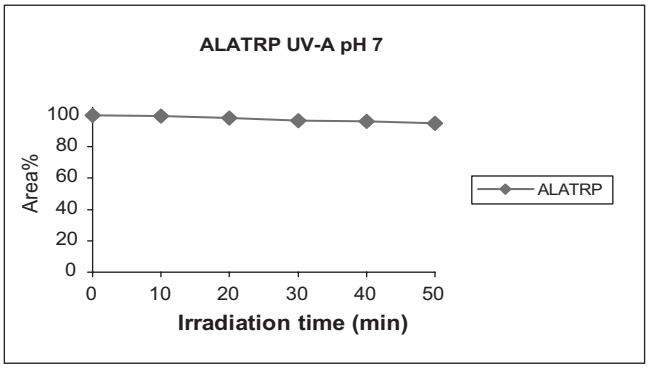


Fig. 10: Photodegradation kinetics of ALATRP at pH 7, exposed to UV-A irradiation

## 3. Experimental

### *3.1. Instrumentation*

The development and first experiments of the described screening method were reported earlier (Schröder and Surmann 2006). The HPLC (Merck/Hitachi) instrument was equipped with a model series L-6200A intelligent pump, Rheodyne 7125 injector with a 20 µl loop and a L-4250 UV-VIS-Detector. The second pump was a HPLC Bischhoff pump. For analyses the wavelength was set at 254 nm. The chromatographic accumulation and separation were performed on two RP $_{18}$ Phenomenex Aqua columns (30 × 4.6 mm, column 1; 100 × 4.6 mm, column 2) which were built in the two Valco 10-port valves. The pH range of the columns is limited between pH 2–10.

The homemade photochemical reactor was equipped with knitted Tefzel (ETFE) reaction coils (10 m × 0.254 mm I.D.). As light source an UV-A TL8W/05 Philips low pressure mercury vapour lamp was used. The conventional heather was used, to hold the temperature between 35–36 °C. For temperature control a digital temperature sensor was installed in the middle of the photoreactor, directly above the capillary.

### *3.2. Materials and reagents*

Hydrochlorothiazide (HC) was obtained from Fagron and promethazine hydrochloride from Caelo. Alany'tryptophane, tryptophane, phosphoric acid 85%, propanesulfonic sodium salt monohydrate and sodium hydroxide were purchased from Fluka. Acetonitrile was of HPLC grade from Sigma Aldrich. Water was destilled and ultrafiltrated.

#### *3.2.1. Buffer solution*

For phosphoric buffer solution, 0.49 g phosphoric acid was dissolved in 900 ml pure water. The pH of the buffer was then titrated to pH 3 or pH 7 with 0.1 mol/l sodium hydroxide. The volume made up to 1000 ml.

#### *3.2.2. Standard solution*

Standard solution of HC was prepared dissolving 16.7 mg of HC in 10.0 ml of the phosphate buffers (5 mmol/l, pH 3, pH 7) and diluting these solution with phosphate buffer to the final concentration of 33.4 µg/ml. For the mixed solution the concentrations were fixed for HC 33.4 µg/ml and for ALATRP 30.96 µg/ml (equimolar to HC).

For the standard solution of PMZ 18 mg were dissolved in 10.0 ml of the phosphate buffer (5 mmol/l, pH 3, pH 7) and diluting these solution with phosphate buffer to the final concentration of 36 µg/ml. For the mixed solution the concentration were fixed for PMZ 36 µg/ml and for ALATRP 30.96 µg/ml (equimolare to PMZ).

All solutions were protected from light with aluminium foil to avoid photochemical degradation of drug and peptide.

### *3.3. Chromatographic conditions for hydrochlorothiazide*

The mobile phase of the isocratic pump 1 was aqueous phosphate buffer pH 3 or pH 7. For the pump 2 a gradient at a flow rate of 1 ml/min with the following profile was used. Eluent A was acetonitrile and B was phosphate buffer pH 3 or pH 7.

### *3.4. Chromatographic conditions for Promethazine*

The mobile phase of the isocratic pump 1 was aqueous phosphate buffer pH 3 or pH 7. Eluent A of pump 2 was an aqueous solution of 0.043 mmol/l potassium hydrogen sulfate and 1 mmol/l propanesulfonic sodium salt monohydrate. Eluent B was acetonitrile with 1 mmol/l propanesulfonic acid sodium salt monohydrate. A gradient at a flow rate of 1 ml/min with the following profile was used.

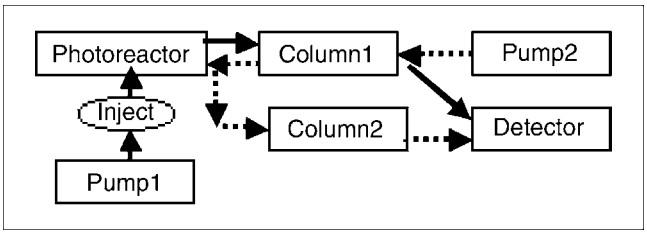


Fig. 11: Schematic diagram of the HPLC system; buffer →; organic phase ·····>

**Table 1: Gradient profile for HC analysis**

| Time (min) | 0.0 | 2.9 | 26.0 | 36.0 |
|---|---|---|---|---|
| Buffer (%) | 100 | 100 | 75 | 50 |
| Acetonitrile (%) | 0 | 0 | 25 | 50 |

**Table 2: Gradient profile for PMz analysis**

| Time (min) | 0.0 | 11.5 | 19.0 | 22.5 | 33.0 | 36.0 |
|---|---|---|---|---|---|---|
| | | 5 | 0 | 5 | | |
| Buffer (%) | 100 | 100 | 85 | 70 | 43 | 43 |
| Acetonitrile (%) | 0 | 0 | 15 | 30 | 57 | 57 |

### *3.5. Chromatographic system*

The system was operated as shown in Fig. 11. Initially, the valves were seated at the position "Load". The buffered solution of pump1 delivered to the photochemical reactor and afterwards the column1 at a flow rate of 1 ml/min. The sample solution was injected from a Rheodyne injector. After 20 s, if the drug has reached the photoreactor the pump 1 was stopped for irradiation. After irradiation the system was started to chromatography.

HC, ALATRP and its photoproducts were adsorbed onto column 1. After 2.9 min the valves were changed in the "Inject" position. The gradient pump 2 delivers to the column 1 and column 2 at a flow rate of 1.0 ml/min. HC, ALATRP and degradation products retained on column 1 were desorbed by delivering the mobile phase in a back-flushing mode.

The method used for PMZ/ALATRP investigation contains a change in switching parameters. During collection of PMZ, the ALATRP eluted from column 1 and was monitored by the UV detector. The switching time was set to 11.5 min. After the valves were switched the gradient pump 2 delivers eluent to column 1 and column 2 at a flow rate of 1.0 ml/min. PMZ and degradation products retained on column 1 were also desorbed by delivering the mobile phase in a back-flushing mode (Tables 1, 2).

**References**

Davies MJ (2004) Reactive species formed on proteins exposed to singulet oxygen. Photochem Photobiol Sci 3: 17–25.

Han KD (2000) Increased phototoxicity of hydrochlorothiazide by photodegradation. Photodermatol Photoimmunol Photomed 16: 121–124.

Horio T, Miyauchi H, Asada Y, Aoki Y, Harada M (1994) Phototoxicity and Photoallergenicity of quinolones in guinea pigs. J Dermatol Sci 7: 130–135.

Moore DE (2002) Drug induced cutaneous photosensitivity: Incidence, mechanism, prevention and management. Drug Safety 25: 345–372.

Neumann N, Blotz A, Wasinska-Kempa G, Rosenbruch M, Lehmann P, Ahr HJ, Vohr HW (2005) Evaluation of phototoxic and photoallergic potential of 13 compounds by different *in vitro* and *in vivo* methods. Photochem Photobiol B, Biology 79: 25–34.

Schröder S, Surmann JP (2006) Phototoxicity testing by online irradiation and HPLC. Anal Bioanal Chem 386: 1695–1700.

Swasano R, Tamat SR, Moore DE (1983) Photolytic decomposition of hydrochlorothiazide. J Pharm Sci 72: 180–183.