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Determination of glafenine in dosage forms and serum by thin layer densitometry and high performance liquid chromatography

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Abstract

New thin layer densitometry and high performance liquid chromatography (HPLC) methods are described for quantitative determination of glafenine in dosage forms in the presence of its photo-degradation products and in serum in the presence of its metabolites. Mobile phases consisting of toluene–isopropyl alcohol–dimethylformamide–water (18:3:1:0.5) and methanol–water–phosphoric acid (80:120:0.5) are found to be efficient for reasonable separation and adequate resolution of glafenine from associated substances by thin layer chromatography (TLC) and HPLC techniques, respectively. The methods are used for the study of glafenine purity, stability, bioavailability, bioequivalence and tablet dissolution rate. The results obtained by TLC and HPLC techniques are in good agreement and offer the advantages of reproducibility and accuracy. © 1997 Elsevier Science B.V.

Keywords: Thin layer densitometry; High performance liquid chromatography; Glafenine assay; Glafenine photodegradation products; Glafenine metabolites; Tablet dissolution rate; Bioequivalence; Bioavailability

1. Introduction

Glafenine [2,3-dihydroxypropyl-*N*-(7-chloro-4-quinolyl) anthranilate] is an analgesic drug that has been used for the relief of all types of pain [1]. Methods reported for quantification of glafenine are few and those published have been based on non-aqueous visual titrimetry [2], potentiometry [3], gravimetry by precipitation with bismuth iodide [4] and polarography [5]. First derivative and differential derivative spectrophotometry [6] and fluorometry [7] have been also suggested for determining glafenine in the presence of their degradation products. A thin layer chromatographic method (TLC) for identification of glafenine has been reported in the French Pharmacopoeia [8]. High performance liquid chromatography (HPLC) determinations of glafenine [9], its major metabolites [10], and pharmacokinetics application for determining glafenine in serum [11] have been suggested.

The present work describes TLC densitometry and HPLC methods for determination of glafenine in dosage forms and serum. Associated photo-decomposition impurities are detected and identified. Tablet dissolution rate and bioequiva-

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lence of glafenine tablets of different origins are also monitored. The results obtained by both TLC and HPLC techniques are in good agreement.

2. Experimental

2.1. Equipment

All TLC densitometric measurements were carried out at 360 nm using a Desaga densitometer CD-60 chromatogram (Desaga, Heidelberg) with a slit width of 2 mm and slit height of 10 mm. The HPLC measurements were made at ambient temperature on an HPLC system consisting of: solvent delivery pump (Waters M 600); column, μBondapak C_{18} (250 mm $\times$ 4.6 mm i.d.) with a particle size of 5 μm; flow rate, 1.5 ml min^{-1}; injector, universal LC injector (model U6k); detector, UV at 360 nm (Lambda-Max model 481); data module, Waters model 730; system controller, programmable model 721; chart speed, 1 in. min^{-1}; sensitivity, 0.05; pump pressure, 2000 psi; run time, 10 min; injection volume, 10 μl.

Tablet dissolution measurements were made according to 1 USP XXI method using a Pharma Test (PTW1) dissolution instrument (Hainburg-Germany). The ultraviolet measurements were made on a Perkin Elmer Lambda 15 UV/Vis spectrophotometer using 1.00 cm quartz cuvettes. Infrared spectral measurements were carried out using a Shimadzu IR-470 using KBr pellets. Mass spectrometric measurements were made on an Autospec Q spectrometer (Fison AG Analytical) under the conditions: ionization mode, EI positive; source temperature, 200°C; electron energy, 70 eV; trap current, 300 μA; scan time, 5 s per decade; and sample introduction method, solid probe.

2.2. Reagents and materials

The TLC stationary phase used was 60 F precoated 20×20 cm^2 silica gel plates (Merck, Darmstadt) (thickness, 0.2 mm) and activated for 10 min at 50°C just before using. All solvents used were of chromatographic grade. The TLC mobile phase used throughout was a mixture consisting of toluene–isopropyl alcohol–dimethylformamide–water (18:3:1:0.5, v/v/v/v). For the ascending technique, one dimension in a heavy borosilicate glass trough chamber (20 cm long $\times$ 10 cm wide $\times$ 40 cm high) at normal saturation for 1 h, development time of 40 min, and a run distance of 13 cm were utilized.

The HPLC mobile phase was methanol–water–phosphoric acid (80:120:0.5) to which 0.1 M aqueous NaOH solution was added to give a pH of 3.5. Before use the eluent was degassed by sonication for 15 min.

A standard 10^{-2} M glafenine solution was prepared by dissolving 374 mg of pure glafenine in 100 ml of the eluent. Dilute solutions (10^{-3}–10^{-5} M) were prepared by appropriate dilutions with the eluent. All solutions were freshly prepared, stored in brown bottles, and kept in a refrigerator when not in use.

Experimental rats of the same family (Sprague-Dawley), sex male, age $\sim$6 months, and weight 180–200 g were used. They were housed in stainless steel cages under optimum conditions for 1 week before drug administration to assure that they were healthy. They were supplied with a conventional diet and drinking water.

2.3. TLC and HPLC determination of glafenine

About 50 ml TLC mobile phase was poured into the chromatographic tank which was covered and left to saturate for 1 h. The plates of the silica gel were dried at 50°C for 10 min and cooled. 10 μl Standard glafenine solutions (10^{-2}–10^{-5} M) were applied. After air drying, the plate was put into the tank and the tank lid was replaced. The chromatogram was run for about 40 min. The plate was removed and the solvent front was marked with a fine pencil point. The chromatogram was dried and the R_f values were detected under a UV lamp. The area of the glafenine spot (R_f 0.37) was measured using a Desaga densitometer and a calibration graph was plotted and used for determination of unknown glafenine concentrations.

For HPLC measurements, aliquots (10 μl) of 10^{-2}–10^{-5} M standard glafenine solutions were

injected into the HPLC chromatograph and the peak area at R_t 7.65 due to glafenine was measured and plotted against the concentration. The calibration graph was used for subsequent measurements of glafenine in samples of unknown purity.

2.4. TLC and HPLC determination of glafenine in serum

5 ml Glafenine (80 mg ml^{-1}) solution in 1% carboxy methyl cellulose (CMC) was given orally in equal portions by means of curved canula to 6 rats of the same sex, age and family. Blood samples (0.5 ml) from each rat were collected from the tail vein at time intervals of 30 min for 3 h and mixed. The serum was stored at 4°C or frozen until analysed. To 0.5 ml of each of the serum pool, 3 ml of methanol–aqueous ammonia solution (99.5:0.5) was added, shaken vigorously and centrifuged for 15 min at 300 rpm. The supernatant was transferred to another tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 0.2 ml methanol, filtered on a 0.2 μm Fluorepore filter and a 10 μl of the filtrate was applied to the silica gel plate or injected into the HPLC chromatograph.

2.5. HPLC measurements of glafenine tablet dissolution

Two tablets, each containing 200 mg, produced by El-Nasr (glafenine) and Roussel UCLAF (Glifarelex) were allowed to dissolve in 500 ml of 0.1 M HCl and stirred at a rate of 100 rpm using the standard dissolution apparatus. The amounts of glafenine dissolved were determined by spectrophotometric and HPLC methods. For spectrophotometric measurements, a filtered portion of the solution (2.0 ml) was diluted to 100 ml with 0.1 M HCl and the absorbance at 343 nm was measured against a blank of 0.1 M HCl in 1.00 cm quartz cuvettes. The absorbance was compared with a calibration graph made using standard glafenine. For HPLC measurements a filtered portion of the solution (2.0 ml) was diluted to 100 ml with 0.1 M HCl and a 10 μl aliquot was injected in the HPLC instrument. The peak area at R_t 7.65 was measured and compared with a standard glafenine.

3. Results and discussion

3.1. Detection of glafenine purity

TLC has never been used for the quantitative determination of glafenine. The TLC method of the French Pharmacopoeia for identification of glafenine [8] involves sample dissolution in chloroform–methanol–water (3:3:0.5), application to a silica gel plate, elution with a mixture of chloroform–methanol–acetic acid–water (85:12:2:1), respectively, and detection under UV radiation where only 2 spots are displayed.

A preliminary study to use this method for quantitative densitometric determination of some glafenine powders and formulations shows only two unresolved overlapped spots at R_f 0.41 (major) and R_f 0.38 (minor). The extent of overlapping is inconsistent and depends on the concentration and volume of the test solution applied to the plates. The main spot due to glafenine constitutes a mean of 92% of the total concentration (Fig. 1). This level is less than the pharmacopoeial acceptance limit ($>97\%$). Attempts to effect resolution of the two spots were made using different stationary phases (silica gel, cellulose and alumina) and various mobile phases. With alumina, the rate of solvent migration was significantly slow, whereas with cellulose the spots were spread over a wide area. Silica gel provided the best compromise for fast rate of solvent migration and regular spots. Scanning of the chromatograms at different reflection wavelengths revealed maximum intensity at 360 nm.

Upon using a mobile phase consisting of toluene–isopropyl alcohol–dimethylformamide–water (18:3:1:0.5) four resolved spots (Fig. 1) were detected at R_t 0.52, 0.46, 0.37 and 0.30 for a certified glafenine sample (purity $>97\%$). Densitometric quantification revealed that the spots at R_f 0.52, 0.46, 0.37 and 0.30 are in a ratio of 0.6, 1.3, 97.0 and 1.1%, respectively. These data indicate high resolution capability and remarkable efficiency of the new mobile phase for displaying

species that are not detected by the standard French Pharmacopoeia method.

In an attempt to identify the nature and composition of the four detected spots several chromatograms were prepared and spots of the same R_f value were scratched from the plate, collected together, dissolved in chloroform, filtered and identified by UV, IR and mass spectrometry (MS). Spots appearing at R_f 0.52, 0.46, 0.37 and 0.30 in the glafenine TLC chromatograms showed m/z values of 312, 279, 372 and 441 in their mass spectra, respectively. The presence of the compounds shown in Fig. 2 as photo-decomposition products has also been confirmed by UV and IR spectrometry. It was difficult, however, to identify the spot appearing at R_f 0.30 (m/z 441).

These data were confirmed by HPLC measurements using a mobile phase consisting of methanol–water–phosphoric acid (80:120:0.2; adjusted to pH 3.5 with 0.1 NaOH) on the same samples. Four signals appeared (Fig. 3) at R_t 7.65, 6.96, 5.17 and 3.49 with areas of 96.9, 1.8, 0.8 and 0.5% ($n = 5$) assigned to glafenine and its photo-decomposition products, respectively. The TLC and HPLC chromatographic methods developed in this study were satisfactorily used for stability indicating assays of the drug and to confirm and to monitor purity of glafenine powders during production.

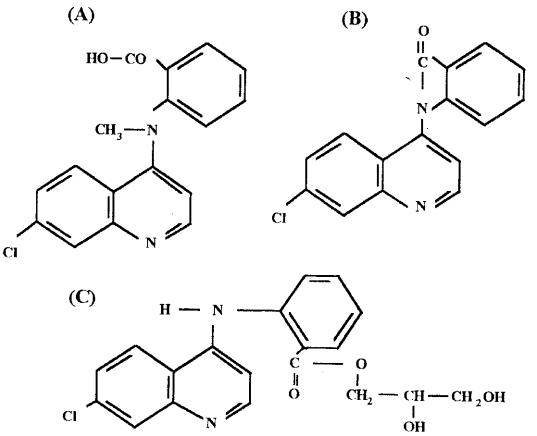

Fig. 2. Chemical structures of the TLC identified spots of pure glafenine. Spot (A) at R_f 0.52 (m/z 312), spot (B) at R_f 0.46 (m/z 279) and spot (C) at R_f 0.37 (m/z 372).

3.2. Determination of glafenine in dosage forms

TLC densitometry detects glafenine quantities of the order of 3.8 μg ml^{-1}. Quantitative TLC determination of glafenine in dosage forms by measuring and evaluating the glafenine spot (R_f 0.37) on the treated chromatograms does not present any problems. It is known that in TLC densitometry the relation between absorbance of a compound and its concentration is not linear and

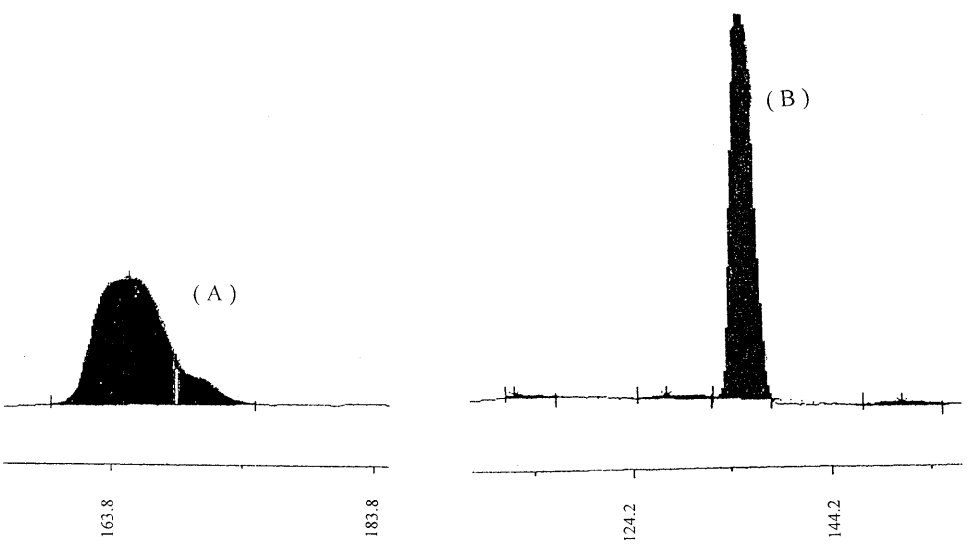

Fig. 1. Typical TLC spectro-densitometric peaks of glafenine using the mobile phases of: (A) French Pharmacopoeia (chloroform–methanol–water (3:3:0.5)) and (B) the proposed method (toluene–isopropyl alcohol–dimethylformamide–water (18:3:1:0.5)).

is best described by the Kubelka-Munk equation [12]. The use of an electronic linearizer in the densitometer gives directly the linear relationship between the peak area of the zone and concentration. It is possible to linearize all curves obtained with various SX values, where S is a coefficient representing the degree of scattering per unit thickness of the layer plate and X is a coefficient representing the degree of absorption per unit thickness of the thin layer plate. The lower limit of detection, defined as a signal value at a signal-to-noise ratio of 3:1, is 1 μg ml^{-1} and the mean S.D. is $\pm 1\%$. Results with an average recovery of 98.5% and a mean S.D. of 1.5% ($n = 5$) of the nominal are obtained with various glafenine dosage forms.

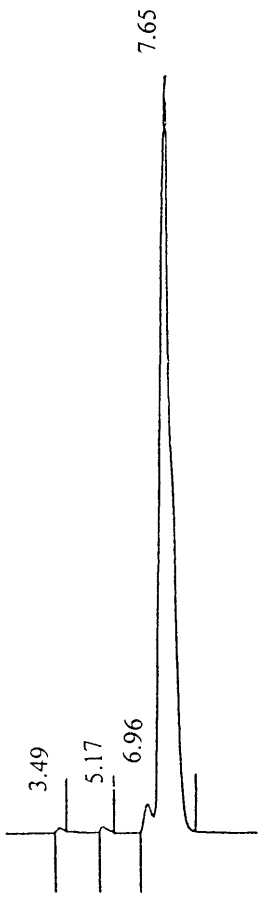

Fig. 3. Typical HPLC chromatograms of glafenine using a mobile phase consisting of methanol–water–phosphoric acid (80:120:0.2; adjusted to pH 3.5 with 0.1 M NaOH).

Using HPLC the peak area-concentration graph indicates a lower limit of detection in the order of 5 μg ml^{-1}. The S.D. is $\pm 1\%$. The linearity holds over the concentration range 0–100 μg ml^{-1}. Determination of glafenine in various dosage forms by monitoring the peak area at R_t 7.65 shows an average recovery of 98.7% and a mean S.D. of $\pm 1.2\%$ ($n = 5$) of the nominal.

The accuracy and precision of both the TLC and HPLC methods were evaluated by determining five replicate samples containing 10 μg ml^{-1} glafenine. The average recoveries were taken as the accuracy of the methods while the precision was estimated by calculating the intra and inter-day relative S.D. (R.S.D.). Mean recoveries of 98.7 and 98.5% ($n = 5$) were obtained by the proposed TLC and HPLC methods, respectively. The intra- ($n = 5$) and inter-day ($n = 15$) R.S.D. were 1.2 and 2.0% in TLC and 1.5 and 1.8% in HPLC, respectively. These results were statistically evaluated by Student's t-test and the variance ratio f-test. Student's t-test values obtained at the 95% confidence level exceed the theoretically tabulated values indicating no significant difference between the two methods. The f-test also showed that there is no significant difference between the precision of the two proposed methods.

3.3. Determination of glafenine in serum

To check the validity of the proposed TLC and HPLC assay methods in pharmacokinetics we monitored the fate of glafenine in serum using blood samples collected at various time intervals (30 min) from 6 rats who received a total of 400 mg glafenine. The TLC chromatograms were identified and compared with authentic standards. The results revealed the presence of glafenine, hydroxy glafenine and glafenic acid at R_f values of 0.37, 0.40 and 0.55, respectively. By using HPLC four main peaks (Fig. 4) at R_t 7.60, 6.10, 4.80 and 2.20 in all chromatograms were detected. These peaks are assigned to glafenine, glafenic acid, unidentified species and hydroxy glafenine, respectively as confirmed by comparison with standard samples.

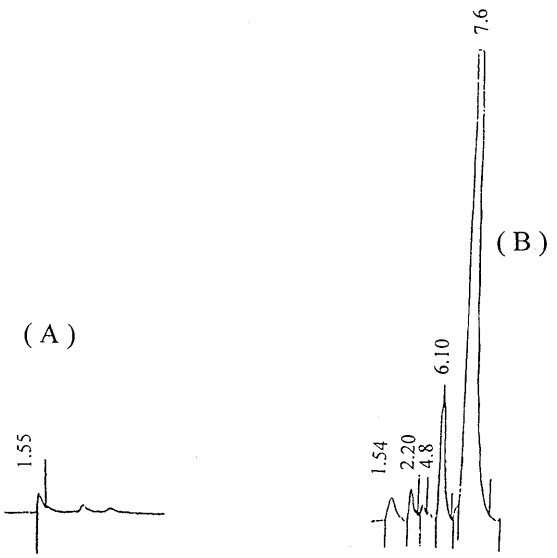

Fig. 4. HPLC chromatograms of extracts from (A) plasma control (blank) of rats and (B) plasma of 6 rats after 30 min of oral intake of a total of 400 mg glafenine.

The bioequivalence of glafenine produced by a local pharmaceutical company (El-Nasr) and that by an international company (Roussel UCLAF) was measured and compared. This was carried out by monitoring the concentrations of glafenine and glafenic acid in the serum of two sets of rats (6 rats each) after oral administration of a total of 400 mg to each set of the glafenine products of the two companies. Use of drug in solution eliminates any effect of the first dynamic step for bioavailability, namely, liberation of the drug from a dosage form and its dissolution in body fluids and induces a more uniform and enhanced bioavailability [13]. Fig. 5 shows typical concentration–time profiles of glafenine in the serum of rats as measured by HPLC methods. This figure also roughly shows the rates of glafenine absorption, disposition, metabolism and excretion.

It can be seen that the concentration level of glafenine in the serum increased with time to reach a maximum after 2 h then decreased gradually due to its conversion into its metabolites. On the other hand glafenic acid concentration increased in the serum with time to reach its maximum value after 1.5 h then gradually decreased due to excretion in urine. After 2.5 h glafenine in the serum reached its minimum concentration level and after 3 h all glafenic acid was excreted in the urine. It may be noticed that both Roussel UCLAF and El-Nasr glafenine behave similarly and display profiles with a similar pattern. The blood level profiles following administration of the two dosage forms are within an expected statistical variance and are superimposed. The area under the average serum drug–time curves can be used as an index for comparing the bioequivalence [13]. Relatively high bioequivalence of the Roussel drug compared to the El-Nasr drug (by a factor of 1.2) was obtained.

3.4. Measurements of glafenine tablet dissolution

A glafenine tablet dissolution profile was made in 0.1 M HCl using a rotating basket dissolution test apparatus at 37°C and 100 rpm. The amount of glafenine dissolved with time was monitored by HPLC and confirmed by UV measurements at 343 nm. Results obtained from dissolution experiments measured with HPLC using 200 mg tablets

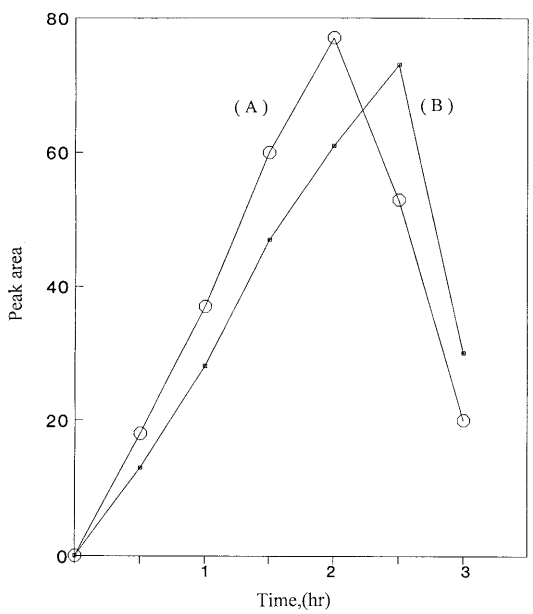

Fig. 5. Concentration–time profile of glafenine in the plasma of 6 rats after an oral intake of a total of 400 mg of (A) Glifarelex (Roussel UCLAF) and (B) Glafenine (El-Nasr).

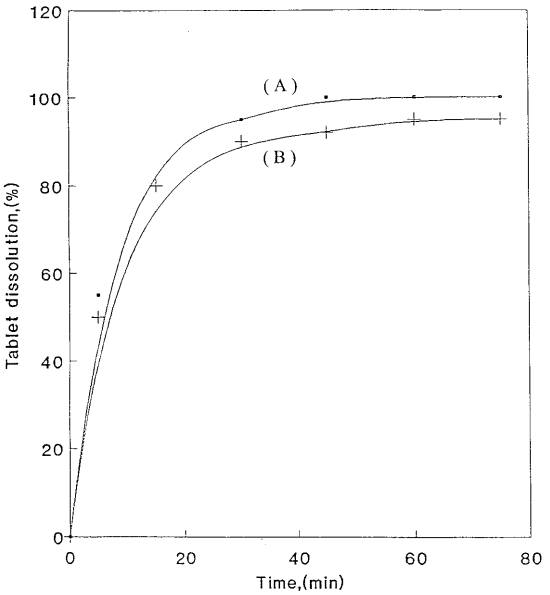

Fig. 6. Dissolution profiles of 200 mg tablets of (A) Glifarelex (Roussel UCLAF) and (B) Glafenine (El-Nasr).

produced by El-Nasr (Glafenine) and Roussel UCLAF (Glifarelex) are shown in Fig. 6. The data were used for quantitative description of the dissolution process as a function of time. A rearranged form of the Hixso-Crowell cube-root equation [14] was used to describe the dissolution process: $f = 1 - (1 - Kt)^3$, where f is the fraction dissolved, t is the time, and K is an experimentally determined constant which depends upon the initial particle size and the solubility of the drug among other things. In the present work, the values of the dissolution rate constants K were determined. In accordance with the above equation the data were plotted as $(1-f)^{1/3}$ versus t and K was obtained directly from the slope. The linearity of this plot was excellent ($r^2 = 0.996$) over the experimental range of f from 0–0.96. The least-squares slope in this range yielded $K = 0.45$ and 0.55 for Roussel and El-Nasr glafenine tablets, respectively.

In conclusion, this work demonstrates that the proposed TLC densitometry and HPLC methods can be used for quantification of glafenine over the concentration levels 0–100 μg ml^{-1}. Both techniques were satisfactorily used for determining drug purity, stability, bioavailability, bioequivalence, metabolites and tablet dissolution.

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