Journal of Chromatography, 363 (1986) 372–381
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 741

Note

E–Z Isomerism of progabide in polar solvents

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(Received April 21st, 1986)

Progabide, 4-[(4-chlorophenyl)(5-fluoro-2-hydroxyphenyl)methyleneamino]-butanamide, is an anti-epileptic drug (Gabren®) possessing an intrinsic and highly specific agonist action on γ-aminobutyric acid (GABA)-ergic receptors[1,2]. Its structure is shown in Fig. 1. Spectral analysis has demonstrated the existence of an hydrogen bond between the imine function and the hydroxyl group on ring A, resulting in the *E*-configuration of the side chain with respect to ring A[3]. An X-ray crystallographic study has confirmed the *E*-configuration and its coplanarity with ring A[4]. Steric interactions, mainly between H-6 and H-2′ (6′) and between H-2′ (6′) and the methylene group of the side chain adjacent to the imine nitrogen, force ring B to adopt an orthogonal conformation (Fig. 1).

Progabide is an amphoteric molecule. The pK_a values for the transitions from the immonium cation to the neutral form and from the neutral form to the phenolate anion are 3.35 and 12.80, respectively. In addition, the existence of a phenol-imine/quinone-enamine tautomerism has been demonstrated[3]. Progabide hydrolyses rapidly in acidic and basic solutions and has maximum hydrolytic stability at around pH 7 (Fig. 2)[5–7].

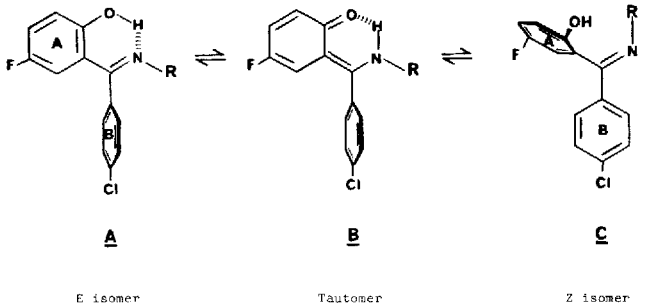

Fig. 1. *E–Z* Isomerism and tautomeric equilibrium of progabide. R = $(CH_2)_3CONH_2$.

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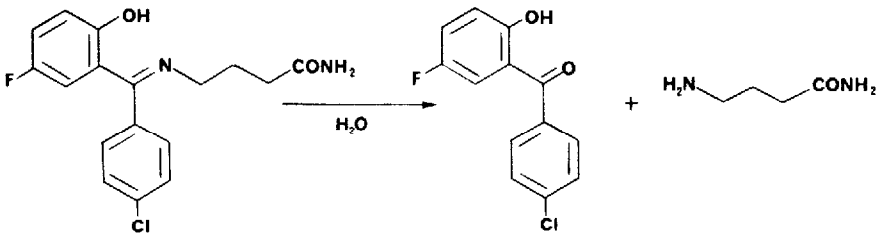


Fig. 2. Hydrolysis of progabide.

The purity of the bulk drug is routinely assessed by reversed-phase high-performance liquid chromatography (HPLC). In the course of the validation of this assay, we have observed an extraneous peak, the size of which depended on the polarity of the solvent used for dissolution of progabide prior to chromatography as well as on the polarity of the mobile phase. A study was therefore undertaken to elucidate the structure of this compound derived from progabide.

EXPERIMENTAL

High-performance liquid chromatography

The HPLC equipment consisted of a Model 340 System Organizer, a Model 110A pump, a model 165 rapid scanning UV/VIS detector and a Model 45 Data System/Controller, all from Beckman Instruments France (Gagny, France). The chromatographic column (150 mm × 4.6 mm I.D.) was laboratory packed with 5-μm Nucleosil C_{18}. The mobile phase was a mixture of 45% (v/v) acetonitrile, 15% ethanol and 40% aqueous buffer (500 ml of 0.1 *M* ammonium monohydrogenphosphate, 200 ml of 0.1 *M* citric acid, pH 6.4, diluted to 1 l with deionized water). The flow-rate was 1.6 ml/min and the temperature was ambient (22 ± 1°C). The compounds investigated were detected at 254 nm and the UV spectra were obtained on-line without interruption of the flow.

Solutions of progabide were prepared at a concentration of 1 mg/ml in acetonitrile, methanol and the HPLC mobile phase, respectively.

HPLC–mass spectrometry (MS)

HPLC–MS with direct sample introduction was performed under the following conditions. The column (250 mm × 0.22 mm I.D.) was laboratory packed with 5-μm μSil C_{18}. The mobile phase was acetonitrile–water (40:60, v/v) and the flow-rates were 8 and 2 μl/min for UV and MS detection, respectively.

A micro-LC Familic-100 (JASCO–Prolabo, Paris, France) equipped with an Uvidec-100 IV UV spectrophotometer (JASCO) was used for column (250 mm × 0.5 mm I.D.) testing (Fig. 7A).

For HPLC–MS a VG-Micromass 7035 double-focusing mass spectrometer (VG Analytical, Manchester, U.K.) equipped with an electron ionization/chemical ionization (EI/CI) source was employed without any modification. The capillary column was fitted through the capillary gas chromatographic introduction system of the mass spectrometer. Spectra were obtained under the following conditions: electron

energy, 70 eV; acceleration voltage, 4 kV; ion-source temperature, 250°C; source pressure, 10^{-5} Torr.

^1H NMR

The NMR spectra were recorded on a Bruker WP 200 spectrometer (Bruker Spectrospin, Wissembourg, France). The ^1H chemical shifts are reported in ppm, using tetramethylsilane ($\delta_{TMS} = 0$ ppm) as internal standard.

RESULTS AND DISCUSSION

HPLC

Fig. 3 illustrates the HPLC separation of the unknown peak and progabide, when the latter compound was dissolved in pure acetonitrile (A) and pure methanol (B). The reference solution was spiked with (4-chlorophenyl)(5-fluoro-2-hydroxyphenyl)methanone, the hydrolysis product of the parent drug.

The UV spectra of progabide and of the unknown peak, obtained by rapid scanning in the course of the HPLC separation, are shown in Fig. 4. The unknown peak has a characteristic spectrum which differs considerably from that of progabide: it does not exhibit absorption bands above 400 nm, a property characteristic of compounds of type B and C (Fig. 1). The UV spectrum of the unknown peak is identical to those of compounds without conjugation of the imine bond with ring A as the reduced product and the *Z*-isomer shown in Fig. 5.

For a mobile phase of given composition, the area of the unknown peak increased with the polarity of the solvent used for the preparation of the reference solution of progabide. The evolution of the unknown peak is illustrated in Fig. 6. The ordinate represents the surface area of the peak divided by the total area count (internal normalization). All three curves exhibit a plateau region. The negative slope of the linear portion of the central curve (mobile phase used as solvent) results from the fact that progabide is partially degraded into the corresponding benzophenone. Since the latter compound possesses a stronger absorbance than progabide itself, the total area count increases and thus the area percentage of the unknown peak apparently decreases. Upon inspection of Fig. 6 it is evident that the rate of formation of the unknown peak is higher in more highly polar solvents.

In order to characterize the unknown peak, attempts were made to isolate it by preparative HPLC. The collected fractions were colourless and turned progressively yellow in the course of solvent evaporation. The mass (EI and CI) and ^1H NMR spectra (in deuterochloroform) of the residue revealed the presence of progabide, (4-chlorophenyl) (5-fluoro-2-hydroxyphenyl)methanone and traces of impurities originating from the solvents used for preparative HPLC. Thus, the unknown peak is transformed into progabide in the course of its isolation, and progabide is hydrolysed into the corresponding benzophenone during evaporation of the solvent fractions.

HPLC–MS

Since all attempts to isolate the compound by preparative HPLC failed, we employed on-line coupling of capillary HPLC and mass spectrometry with direct sample introduction. Representative chromatograms illustrating the separation by

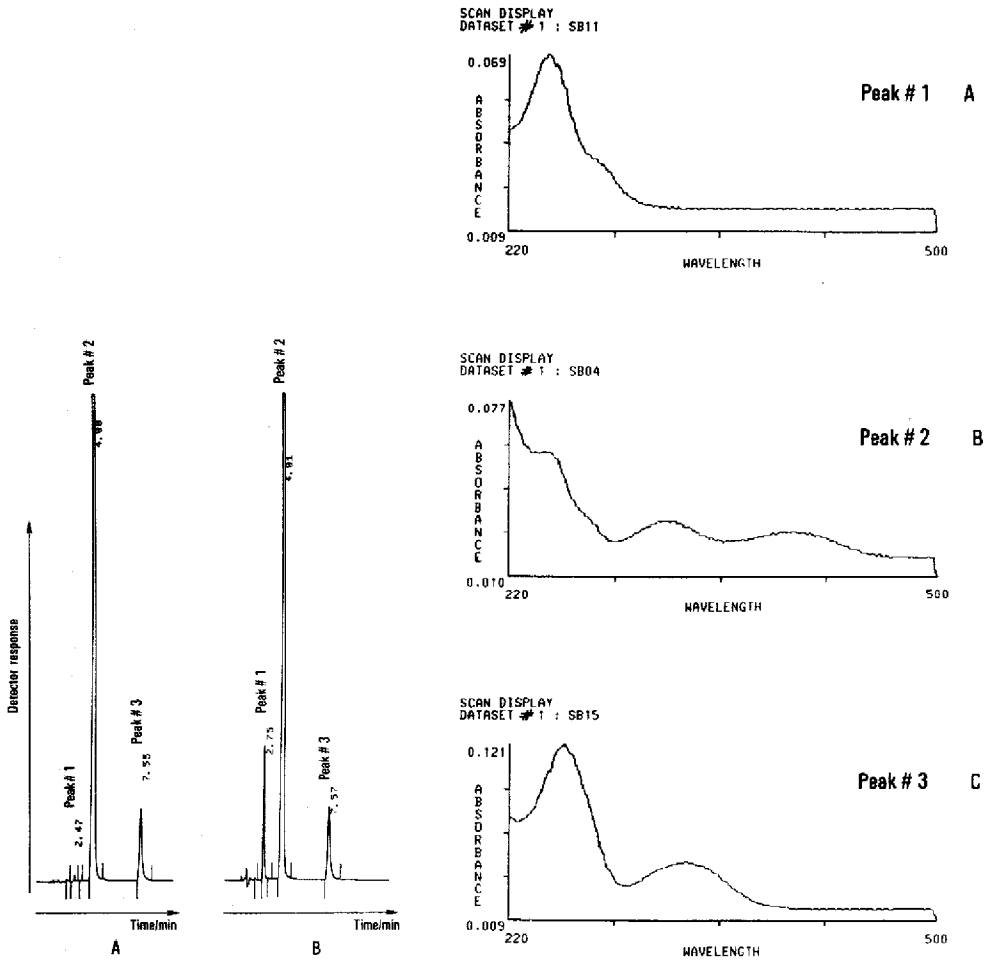


Fig. 3. Chromatogram illustrating the HPLC separation of the unknown (peak 1), progabide (2) and (4-chlorophenyl)(5-fluoro-2-hydroxyphenyl)methanone (3). Solvents used for dissolution of progabide acetonitrile (A); methanol (B). Chromatographic conditions as in Experimental.

Fig. 4. UV spectra obtained by rapid scanning in the course of the HPLC separation: (A) unknown peak; (B) progabide; (C) (4-chlorophenyl)(5-fluoro-2-hydroxyphenyl)methanone. Wavelength in nm.

capillary HPLC with UV and MS detection (total ion current and selected-ion monitoring of $m/e = 251$) are shown in Fig. 7A and B, respectively.

Taking into account the aqueous solvent, the temperature and the vacuum used, the mass spectra of the unknown product and progabide are identical and correspond to that of the hydrolysis product, the substituted benzophenone. For comparison, the spectrum of progabide introduced into the mass spectrometer under the same conditions (solvent and temperature) but without the HPLC column is shown in Fig. 8.

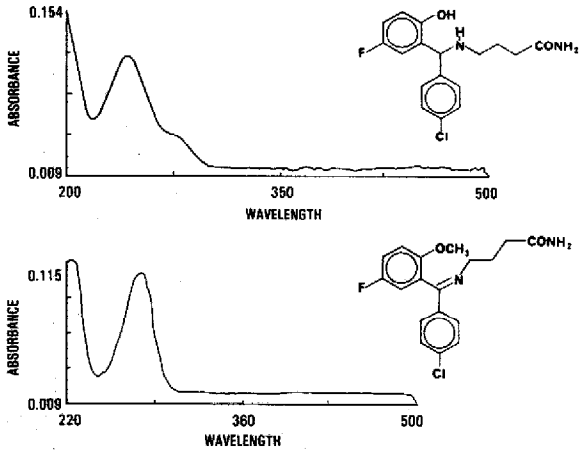


Fig. 5. UV spectra obtained by rapid scanning in the course of the HPLC separation: (A) dihydroprogabide derivative; (B) 2-methoxyprogabide analogue. Wavelength in nm.

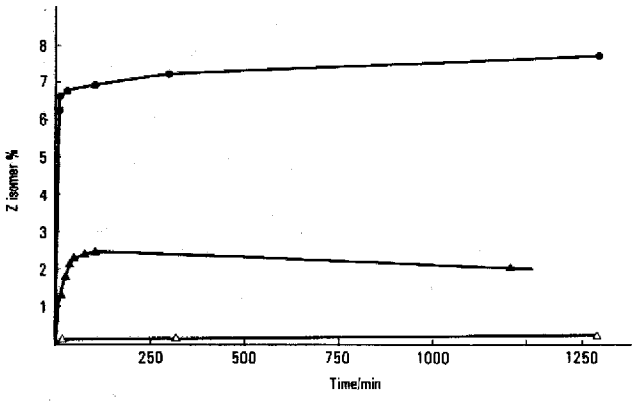


Fig. 6. Kinetics of formation of the unknown product as a function of the composition of the solvent used for preparation of the reference solution of progabide: △—△, acetonitrile; ▲—▲, mobile phase; ●—●, methanol.

^1H *NMR*

The *in situ* analysis of progabide dissolved in deuterated methanol confirmed the presence of approximately 6% of an impurity (Fig. 9). The spectrum of this impurity did not correspond to any of the known potential impurities of progabide, particularly the benzophenone derivative. The additional doublet of doublets (dd) at 6.86 ppm having a 9-Hz coupling with a fluorine atom corresponds to the H-6 proton of a benzylidene derivative. A detailed interpretation of the ^{1}H NMR spectrum is as follows:

Progabide (94%)

H-6; 6.56 ppm, dd ($J_{6,F}$ = 10 Hz, $J_{6,4}$ = 3 Hz)

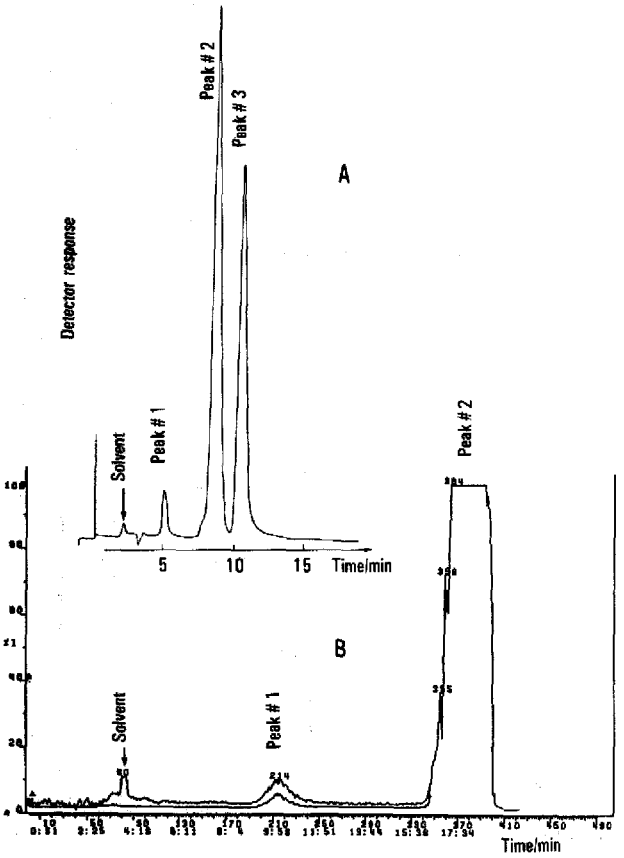


Fig. 7. Chromatograms illustrating the capillary HPLC separation of the unknown product (peak 1), progabide (2) and (4-chlorophenyl)(5-fluoro-2-hydroxyphenyl)methanone (3): (A) UV detection at 254 nm; (B) MS detection (total ion current and selected-ion monitoring of m/e = 251). Chromatographic conditions as in Experimental.

H-3; 7.03 ppm, dd ($J_{3,F}$ = 5 Hz, $J_{3,4}$ = 9 Hz)
H-4; 7.20 ppm, dd ($J_{4,F}$ = $J_{4,3}$ = 9 Hz; $J_{4,6}$ = 3 Hz)
AA'BB' system at 7.42 and 7.75 ppm

Impurity (6%)

H-6; 6.86 ppm, dd ($J_{6,F} \approx 9$ Hz, $J_{6,4}$ = 3 Hz)
AA'BB' system at 7.50 and 7.71 ppm

The paramagnetic shift of proton H-6 in the impurity with respect to the H-6 signal in progabide could be explained by the relative difference in conjugation of the two aromatic rings A and B, as observed for the *E* and *Z* isomers at the imine bond (see Fig. 1 and Table I).

CONCLUSIONS

The above experimental data lead to the following conclusions. (a) Based on the HPLC results: the concentration of the product formed depends on the compo-

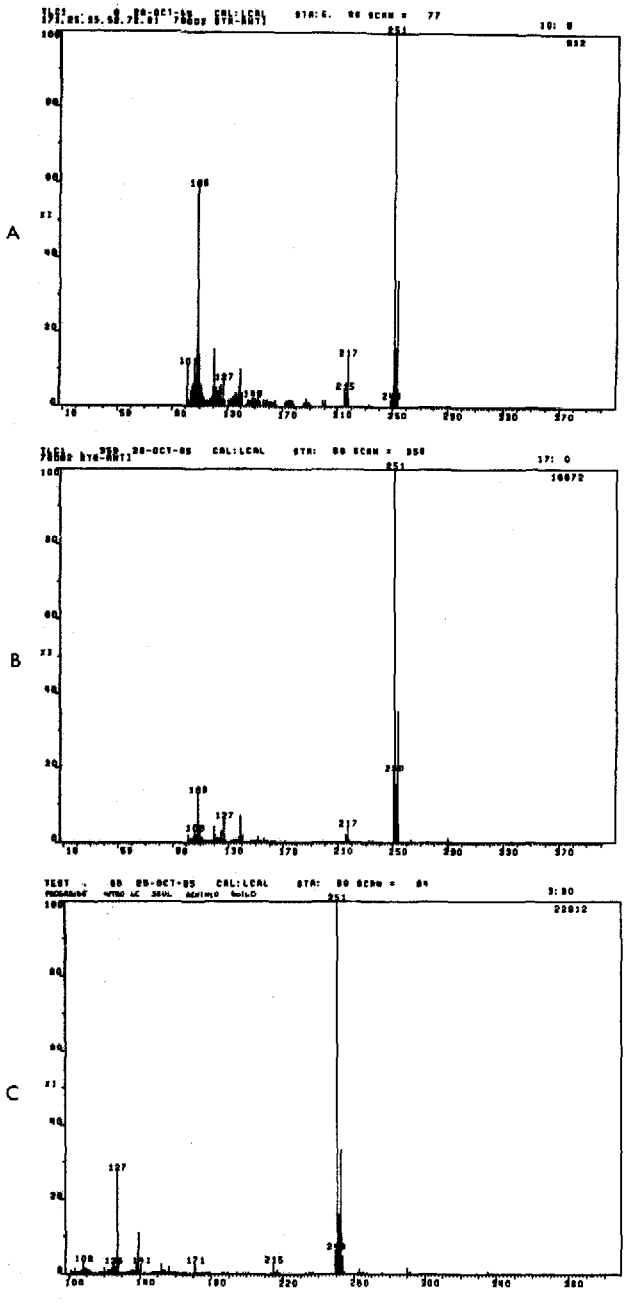


Fig. 8. Mass spectra: (A) of the unknown peak obtained using a mass spectrometer as an HPLC detector; (B) of progabide obtained as in (A); (C) of progabide analysed directly.

sition of the HPLC mobile phase and on the polarity of the solvent used for preparation of the working solutions; the compound investigated is in equilibrium with progabide since it is converted into progabide in the course of its isolation. (b) Based

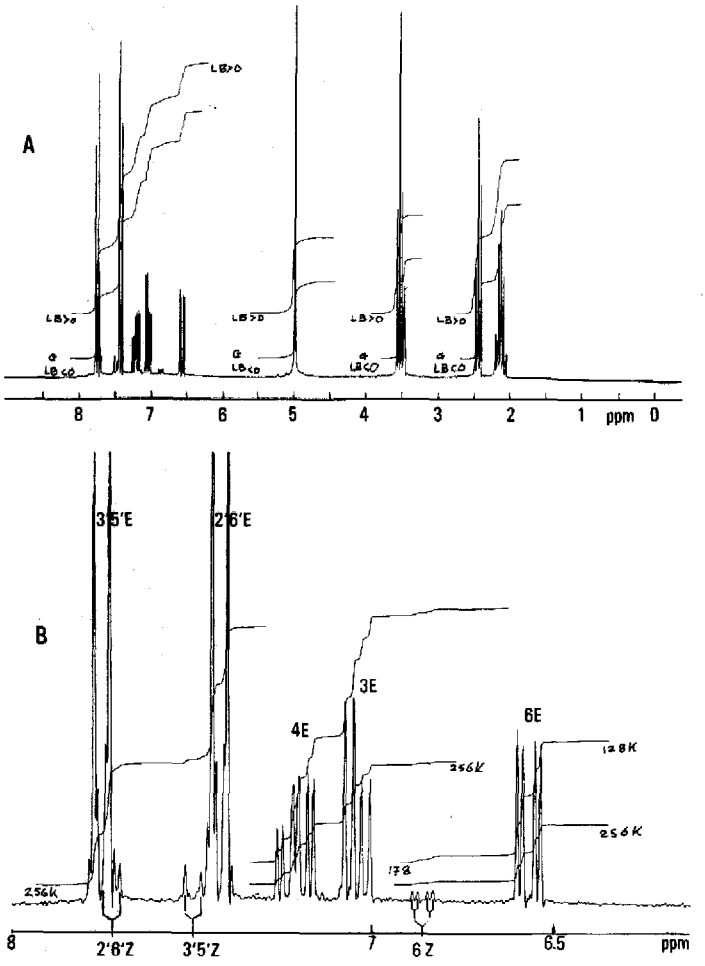


Fig. 9. ^1H NMR spectra: (A) of progabide dissolved in $C^2H_3O^2H$ obtained 24 h after preparation of the reference solution; (B) scale expansion.

on spectral data: the UV spectrum of the product is similar to those of the *Z*-isomers; the mass spectrum is identical to that of progabide; the NMR data indicate the *Z*-isomer.

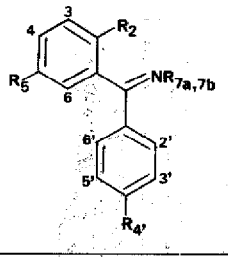
It is suggested that the non-isolable product formed in the course of HPLC analysis of progabide corresponds to the *Z*-isomer of progabide.

E–Z isomerism has also been observed with 2-amino derivatives (Fig. 10). The synthesis of 2-[(2-amino-5-chlorophenyl)phenylmethyleneamino]acetamide gave two isomers (*E* and *Z*) at the imine bond. These products were isolated and characterized. The transformation of the *E*-isomer into the *Z*-isomer, followed by an intromolecular ring closure leading to desmethyldiazepam, has been demonstrated[8].

In a previous paper[3], we have shown by UV spectroscopy the existence of a phenol-imine/quinone-enamine tautomerism and the shift from the phenol-imine towards the quinone-enamine with increasing solvent polarity (Fig. 1: A → B)[3]. The

TABLE I

^{1}H NMR CHEMICAL SHIFTS FOR *E*- AND *Z*-ISOMERS OF PROGABIDE AND ANALOGUES



R_{7a} = CH_2CONH_2 (8)

R_{7b} = CH_2 (8) CH_2 (9) CH_2CONH_2 (10)

R_2	R_5	R_4	R_7	*E/Z*	*Solvent*	*H-3*	*H-4*	*H-6*	*H-2' (6')*	*H-3' (5')*	*H-4'*	CH_2-8	CH_2-9	CH_2-10
NH_2	Cl	H	a	*E*	$(C^2H_3)_2SO$	6.80	7.10	6.52	7.20	7.50	7.50	3.85	–	–
NH_2	Cl	H	a	*Z*	$(C^2H_3)_2SO$	6.80	7.20	6.82	7.70	7.50	7.50	4.00 (1H) 3.72 (1H)	–	–
OH	Cl	H	b	*E*	C^2HCl_3	6.90	7.13–7.28	6.74	7.13–7.28	7.46–7.57	7.46–7.57	3.36	1.98	2.34
OCH_3	Cl	H	b	*Z*	C^2HCl_3	6.92	7.43–7.24	6.97	7.59	7.43–7.24	7.43–7.24	3.31	1.99	2.38
OH	Cl	Cl	b	*E*	C^2HCl_3	6.90	7.22	6.71	7.13	7.52	–	3.36	1.98	2.33
OCH_3	Cl	Cl	b	*Z*	C^2HCl_3	6.93	7.36	6.96	7.53	7.36	–	3.31	1.99	2.37
OCH_3	F	Cl	b	*Z*	C^2HCl_3	6.92	7.10	6.72	7.54	7.29	–	3.31	1.99	2.37
Progabide				*E*	C^2HCl_3	6.91	6.99	6.46	7.13	7.51	–	3.38	1.99	2.34
Progabide				*E*	$C^2H_3O^2H$	7.03	7.20	6.56	7.42	7.75	–	3.50	2.13	2.43
				Z	$C^2H_3O^2H$	*	*	6.86	7.71	7.50	–	*	*	*

* Hidden by the signals of the main *E*-isomer.

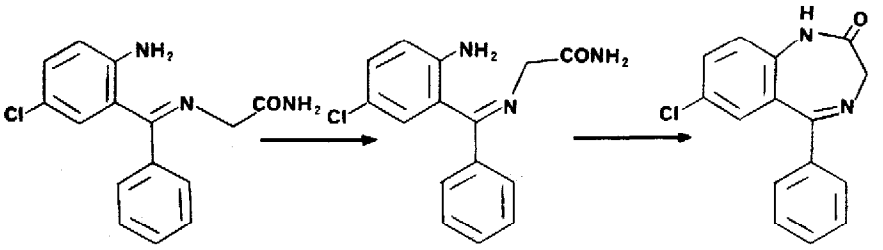
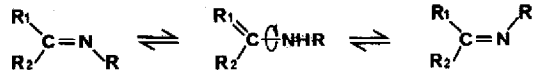


Fig. 10. *E* to *Z* isomerism followed by an intramolecular ring-closure reaction of *E*-2-[(2-amino-5-chlorophenyl)phenylmethyleneamino]acetamide in acidic and neutral media.

concentration of the *Z*-isomer of progabide also increases with the solvent polarity. Thus it can be suggested that progabide isomerizes via an imine–enamine tautomeric mechanism (Fig. 1: A → B → C)[9] (R_1, R_2 = aromatic ring; R = alkyl chain):



This study also shows the critical influence of the choice of the solvent on the accuracy of HPLC assays for substances which may be engaged in tautomeric and isomeric equilibria.

ACKNOWLEDGEMENTS

We would like to thank Dr. Alexander E. Wick for his helpful comments and Jean-Louis Vendé for his excellent technical assistance in the HPLC analyses.

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