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Difference spectrophotometric assay of nitrazepam in tablet formulations

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Abstract—A difference spectrophotometric procedure is described for the assay of nitrazepam in tablet formulations. The method is based on the measurement of absorbance at 282 nm of a solution of the tablet extract in 0·1 M hydrochloric acid (pH 1) relative to that of an equimolar solution in 0·1 M sodium hydroxide (pH 13). The method is precise and selective for nitrazepam in the presence of the tablet excipients and 2-amino-5-nitrobenzophenone, the principal hydrolysis product of nitrazepam. The absence of a constant isosbestic point in the difference spectrum of nitrazepam during hydrolysis in alkaline solution indicates the presence of a previously unrecognized intermediate hydrolysis product.

The advantages of difference spectrophotometry, which is a modified spectrophotometric technique for the selective analysis of certain drugs in the presence of absorbing substances that interfere in a direct spectrophotometric assay, have been amply demonstrated (eg Doyle & Fazzari 1974; Davidson 1976, 1982, 1984a, 1987). The basis of a difference spectrophotometric assay is that an absorbance difference (ΔA) is measured between two equimolar solutions of the absorbing analyte in the presence of different reagents that reproducibly alter the spectral properties of the analyte. Provided that the absorbance of the other absorbing interferents is not affected by the reagents, their ΔA is zero and their contribution to the measured ΔA of the sample is eliminated. Of the many reagents that have been used to induce differences in the spectra of drugs, simple aqueous acids, alkalis and buffers have been the most frequently used because many drugs are weak acids or bases whose state of ionization and absorptivity depend on the pH of the solution.

The 1,4-benzodiazepines comprise a class of drug in which several sites of protonation and deprotonation exist, and which exhibit marked changes of spectral properties as a result of variation of pH (Barrett et al 1973). These spectral transformations have been utilized in the simultaneous difference spectrophotometric assay of chlordiazepoxide and its hydrolysis product, demoxepam (Davidson 1984b) and in the assay of diazepam, clonazepam and medazepam (Abdel-Hamid et al 1984). As part of a continuing research programme, the present study was undertaken to develop a difference spectrophotometric assay for nitrazepam and to evaluate its suitability as a stability-indicating assay for the measurement of the reaction kinetics of the hydrolysis of nitrazepam in alkaline solution.

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The principal decomposition reaction of the 1,4-benzodiazepines is hydrolysis to the corresponding benzophenone by cleavage of the 1,2-amide and 4,5-azomethine bonds (Schutz 1982). Several of the benzodiazepines yield an intermediate hydrolysis product: diazepam produces 5-chloro-2-glycyl (methyl)amino-benzophenone in acidic solution by the preferential cleavage of the 4,5-bond whereas oxazepam yields an acidic intermediate product by the preferred cleavage of the 1,2-bond (Han et al 1977a). It has been reported also that the initial hydrolysis step of nitrazepam is at the 4,5-bond and not the 1,2-amide linkage, attributed to a preferential activation for hydrolysis of the azomethine linkage by the nitro group. The intermediate hydrolysis product was detected only in solutions of pH less than the pK_a of nitrazepam, 3·2 (Han et al 1977b).

On the basis of the facts known about the hydrolysis of nitrazepam, it was considered during the initial development of the method that a stability-indicating assay for the measurement of the reaction kinetics of nitrazepam in alkaline solution should be selective for nitrazepam in the presence of 2-amino-5-nitrobenzophenone only. The results of the study showed that although the method was selective for nitrazepam in the presence of the benzophenone, an extra product was present in solutions of nitrazepam hydrolysed at alkaline pH that interfered in the assay.

Materials and methods

Materials. Nitrazepam and 2-amino-5-nitrobenzophenone were gifts from Roche Products Ltd. Hydrochloric acid, 1 M, sodium hydroxide, 1 M, acetate buffer pH 6, 0·1 M and sodium tetraborate, 0·01 M, were prepared from analytical reagent grade substances.

Spectrophotometer. Absorption and difference absorption spectra were recorded in 1 cm silica quartz cells over the range 230–450 nm by using a Perkin Elmer 552 ultraviolet-visible double-beam recording spectrophotometer. The spectral bandwidth was 2 nm, the scan speed 1 nm s^{-1} and response 0·5 s.

Standard solutions. A standard solution of nitrazepam (200 μg mL^{-1}) was prepared by dissolving 20 mg in 10 mL of ethanol and diluting it to 100 mL with water. A 5 mL aliquot was transferred to two 100 mL volumetric flasks containing 10 mL of 1 M

hydrochloric acid and 10 mL of 1 M sodium hydroxide, respectively, and the contents of both flasks were diluted to 100 mL with water. The absorbance of the acidic solution was immediately measured at 282 nm relative to the absorbance of the alkaline solution. The absorbance difference at 282 nm (ΔA_{282}) was corrected if necessary for the absorbance difference of 0·1 M hydrochloric acid relative to 0·1 M sodium hydroxide.

Sample solutions. Twenty tablets were weighed and powdered. A weight of powder equivalent to 10 mg of nitrazepam was shaken for 15 min with 25 mL of 20% aqueous ethanol in a 50 mL volumetric flask and then diluted to volume with water. The extract was filtered through Whatman No. 1 filter paper and the first 10 mL were discarded. The next 20 mL fraction of filtrate was collected and the assay was continued as described for the standard solutions from the words 'a 5 mL aliquot was transferred to two 100 mL volumetric flasks. . . .'

The concentration of nitrazepam in the sample solutions, and hence in a tablet of average weight, was calculated with reference to the ΔA_{282} of the standard solutions and the proportional relationship that exists between the ΔA_{282} and concentration of nitrazepam.

Results and discussion

Development of the method. In acidic solution nitrazepam is protonated on the N-4 atom and in alkaline solution undergoes deprotonation of the N-1 atom. The pK_a values of these dissociations are 3·2 and 10·8, respectively (Barrett et al 1973). The ultraviolet absorption spectra of equimolar solutions of nitrazepam in 0·1 M hydrochloric acid (pH approximately 1), pH 6 acetate buffer and 0·1 M sodium hydroxide (pH approximately 13) are shown in Fig. 1. These solutions which are at pH values at

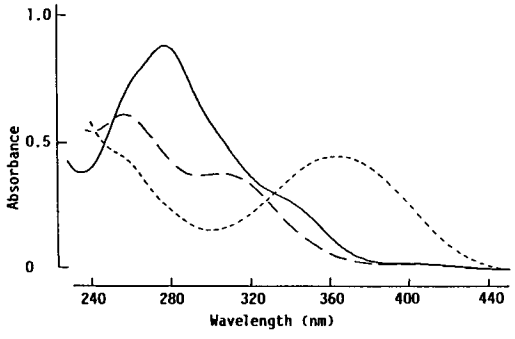

FIG. 1. The ultraviolet absorption spectra of nitrazepam (10 μg mL^{-1}) in 0·1 M hydrochloric acid (pH 1) (——), pH 6 acetate buffer (— —) and 0·1 M sodium hydroxide (pH 13) (– – – –).

least 2 pH units from the pK_a values to give at least 99% of the individual species, produce the positively charged (on N-4), neutral and negatively charged (on the oxygen atom on C-2, as a result of enolization at the 1,2 position) species of nitrazepam, respectively. The spectral shifts observed have been explained by the different states of ionization of the substituents of the two benzene rings of the benzodiazepine molecule (Barrett et al 1973). 2-Amino-5-nitrobenzophenone, the principal hydrolysis product of nitrazepam, exhibits no alteration of its spectrum over the pH range 1-13. This is probably owing to the very weak basic nature of the amino group as a result of the powerful inductive effect of the *p*-nitro group.

The difference absorption spectrum of the solution of nitrazepam at pH 1 in the sample cell of a double-beam spectrophotometer and an equimolar solution at pH 13 in the reference cell shows a wavelength of maximum ΔA (λ_{max}) at 282 nm and a wavelength of minimum ΔA (λ_{min}) at 372 nm (Fig. 2). An

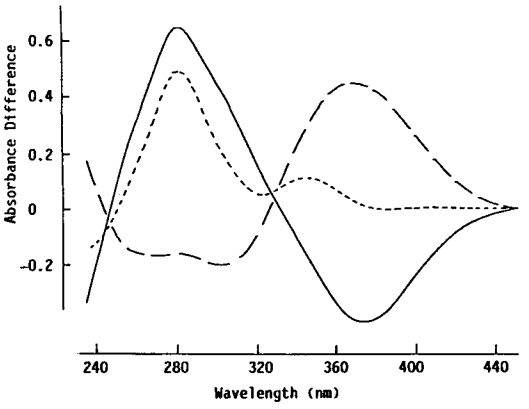

FIG. 2. The difference absorption spectra of nitrazepam (10 μg mL^{-1}): pH 1 solution versus pH 13 solution (——); pH 1 solution versus pH 6 solution (– – – –); pH 13 solution versus pH 6 solution (— —).

isosbestic point (a wavelength of zero ΔA owing to the equal absorptivity of the two species) occurs at 330 nm. The difference absorption spectrum of nitrazepam at pH 13 recorded against the equimolar solution at pH 6 shows a λ_{max} at 368 nm, a λ_{min} at 304 nm and an isosbestic point at 326 nm. The difference spectrum of the pH 1 solution relative to the pH 6 solution has λ_{max} at 282 and 346 nm and an isosbestic point at 252 nm. When recorded under the same conditions, the difference absorption spectrum of 2-amino-5-nitrobenzophenone is identical to that of the solvent baseline. Thus, measurement of ΔA at a number of different maxima and minima is possible. Of these, the ΔA_{282} between pH 1 and pH 13 solutions was chosen because the absorptivity of 2-amino-5-nitrobenzophenone at 282 nm was close to its λ_{min} near 295 nm.

Validation. The proportionality of the ΔA_{282} and the concentration of nitrazepam was checked by measuring the ΔA_{282} of six pairs of solutions at pH 1 and pH 13 containing 0–20 μg mL^{-1}. The linear regression equation calculated by using the method of least squares was $y = 0{\cdot}0632x + 0{\cdot}0004$ (correlation coefficient 0·9996) where y is the ΔA_{282} and x is the concentration in μg mL^{-1}. The rectilinearity of the values and the negligible intercept confirm that there is a proportional relationship between the ΔA_{282} and concentration.

The precision determined on ten pairs of solutions of nitrazepam (10 μg mL^{-1}) at pH 1 and pH 13, calculated as the relative standard deviation, was 0·56%, indicating the excellent reproducibility of the assay.

To assess the selectivity of the procedure when 2-amino-5-nitrobenzophenone is present in the sample solutions of nitrazepam, standard mixtures of nitrazepam and 2-amino-5-nitrobenzophenone were prepared to simulate solutions of nitrazepam that had undergone varying degrees of hydrolysis in the range 0–100%. The results in Table 1 show that even very high

Table 1. Selectivity of the assay in the presence of 2-amino-5-nitrobenzophenone (ANBP).

Composition of mixture				Concn of nitrazepam found	
Nitrazepam (μg mL^{-1})	ANBP (μg mL^{-1})	Hydrol. (%)	ΔA_{282}	μg mL^{-1}	Recovery (%)
10·16	0·00	0	0·638	10·10	99·4
9·14	0·87	10	0·574	9·08	99·3
7·62	2·16	25	0·487	7·71	101·2
5·08	4·33	50	0·324	5·13	101·0
2·59	6·49	75	0·167	2·65	102·3
0·00	8·66	100	0·003	0·05	—

concentrations of 2-amino-5-nitrobenzophenone have no effect on the measured ΔA_{282} of nitrazepam. The selectivity of the procedure was confirmed by examination of the difference spectra of the mixtures, which showed that the λ_{max}, λ_{min} and isosbestic wavelengths of nitrazepam were unaffected by the presence of the benzophenone. The coincidence of the isosbestic wavelength in particular, is excellent evidence of the lack of interference from the benzophenone (Doyle & Fazzari 1974).

To evaluate the suitability of the procedure as a stability-indicating assay for nitrazepam, a solution of nitrazepam in 0·01 M sodium tetraborate (pH 9·18 at 20°C) was heated at 75°C. Aliquots were removed at 10 min intervals for 2 h and assayed for the remaining nitrazepam. The difference spectra of the solutions showed a gradual bathochromic shift of the λ_{min} at 372 nm to about 383 nm, with time, and variations of the isosbestic wavelength over the range 330–342 nm. These observations, which were unexpected, indicate the presence of a previously unrecognized hydrolysis product in addition to 2-amino-5-nitrobenzophenone. The presence of this product which has been the subject of a separate investigation (Davidson et al to be published) renders the difference spectrophotometric procedure unsuitable for use as a stability-indicating assay.

Assay of nitrazepam tablets. A number of nitrazepam tablet formulations from different manufacturers were assayed by the difference spectrophotometric procedure. The results in Table 2 show that the contents of nitrazepam in the tablets were in good agreement with the stated content and with the results obtained by using the assay method of the British Pharmacopoeia 1980. The coincidence of the λ_{max}, λ_{min} and isosbestic wavelengths in the difference spectra of the solutions obtained with the tablet extracts and in those of the standard solutions of nitrazepam is reasonable evidence that the difference spectra of the sample solutions were free of interference from the tablet excipients and that the samples did not contain significant levels of intermediate hydrolysis products.

Table 2. Assay results.

Nitrazepam tablets	Content of nitrazepam			
	This method		B.P. method	
	mg/tablet	% stated	mg/tablet	% stated
Manufacturer A				
Batch 1	5·06	101·3	5·14	102·7
Batch 2	5·02	100·4	4·95	99·0
Manufacturer B				
Batch 1	4·91	98·2	5·00	100·0
Batch 2*	4·79	95·8	4·83	96·6

* Eight-year old sample showing 2-amino-5-nitrobenzophenone by the BP Related Substances test for nitrazepam.

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