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The polarographic and spectral behaviour of some 1,4-benzodiazepine metabolites: application to differentiation of mixtures

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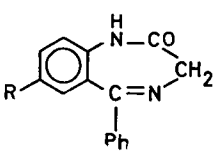
Changes of ultraviolet absorption spectra with pH in solution were used to determine pKa values for four 1,4-benzodiazepine metabolites. 7-Acetamido-nitrazepam, desmethyl-diazepam and the chlordiazepoxide lactam all gave two pKa values, corresponding to protonation in acid and deprotonation of the neutral molecule in alkaline media. 7-Amino-nitrazepam gave three pKa values, the third one being due to an additional protonation in acid media. The spectra are explained by considering them to be superimposed spectra of the two benzene rings, one mono-substituted (band I) and one trisubstituted (band II), within the molecule. Sites of protonation and deprotonation are predicted and the differences in the observed pKa values explained. Differences in the pKa values or the polarographic behaviour between the parent compounds and some of their metabolites are then used to effect novel separations after solvent extractions from aqueous buffered solutions.

Earlier, Barrett, Smyth & Davidson (1973) dealt with an examination of the acid-base equilibria existing in aqueous solutions, pH 1–14, of the 1,4-benzodiazepines, chlordiazepoxide, diazepam, medazepam, nitrazepam, oxazepam and lorazepam. This resulted in the determination of their pKa values and probable sites of protonation and deprotonation. This approach has been extended to some of the metabolites of nitrazepam, diazepam and chlordiazepoxide (I–IV), giving rise to selective solvent



I R = Me CONH-7-acetamido-nitrazepam; II R = NH_2 = 7-amino-nitrazepam; III R = Cl = desmethyl-diazepam; IV R = Cl N → O = chlordiazepoxide lactam.

extraction of diazepam in the presence of desmethyl-diazepam. Berry (1971) has investigated the polarographic behaviour of desmethyl-diazepam and found it identical to diazepam in 0·1 N hydrochloric acid saturated with sodium tetraborate. Differences in the polarographic behaviour of the metabolites I–IV, as compared to their parent compounds, have been used to differentiate mixtures after extraction of parent compound and metabolite(s) at a pH where they all exist as neutrally charged molecules.

MATERIALS AND METHODS

Apparatus

Spectra over the ultraviolet range were recorded in solutions maintained at 20° using a Perkin-Elmer double beam 137 ultraviolet visible spectrophotometer. Matched 1 cm path length silica cells were used and the instrument was flushed with dry nitrogen to eliminate stray radiation effects.

Polarographic curves were recorded using a P.A.R. Model 174 Polarographic Analyser, as operated in the differential pulse mode, in conjunction with a micro Kalousek vessel with a saturated calomel electrode. The dropping mercury electrode used had the following characteristics: outflow velocity $m = 1{\cdot}73$ mg s^{-1}, drop time $t = 4$ s at the potential of the saturated calomel electrode and at a mercury pressure $h = 80$ cm in 1M potassium chloride. For the analyser the controlled drop time was 0·5 s with a modulation amplitude of 100 mV. Potentials are peak potentials.

Reagents

Samples of the benzodiazepines and their metabolites were obtained from the source mentioned in the acknowledgements.

Stock solutions of each drug (about 10^{-3}M) were prepared in Analar methanol and kept in the dark under refrigeration to minimize the possibility of decomposition.

A stock Britton-Robinson (BR) buffer solution (pH approximately 2·0), composed of a mixture of boric acid, phosphoric acid and acetic acid, all 0·04M, was prepared using Analar grade reagents. From this buffer solutions of varying pH were prepared by addition of 0·1M sodium hydroxide solution to the stock solution and measuring the pH on a meter.

Experimental techniques

Experimental solutions were prepared for spectrophotometry by diluting the appropriate amount of stock benzodiazepine solution with the appropriate buffer to give a drug concentration of 5×10^{-5}M. To extend the pH range studied at either end of the scale, M HCl, 0·1M HCl, 0·1M NaOH, and M NaOH were used. The range from about pH 1–13 was scanned for each drug in increments of 1 pH unit to determine the approximate position of each pKa value by observation of spectral changes over the whole range. The region around each pKa value was then studied in more detail using buffers differing by increments of approximately 0·3 pH units. From the spectra obtained, pKa values were evaluated using the Henderson equation (Davidson & Smyth, 1972). The wavelength region scanned was from 200–390 nm. A slow scan speed (8 min for the range) was used and the instrument reference beam contained a blank of buffer solution containing the same amount of methanol as the samples.

In ascertaining the polarographic behaviour of the drug, 2 ml of 5×10^{-5}M benzodiazepine in the appropriate supporting electrolyte was de-aerated by a stream of nitrogen for 3 min and the i—E curve recorded at a rate of 10 mV s^{-1} and modulation amplitude of 100 mV. This slow rate of scanning reduces the effects of hysteresis of the recording instrument.

Polarography was used to monitor the benzodiazepine content of the aqueous phase (5 ml 5×10^{-5}M benzodiazepine buffered at a particular pH or in M sodium hydroxide) in the solvent extraction procedures. The i—E curves of this phase were recorded before and after 10 min shaking with 5 ml of the solvent. In cases where

the benzodiazepines were completely extracted into the solvent, the organic layer was separated, the solvent evaporated off and the residue made up to 5 ml in the appropriate supporting electrolyte before polarographic examination.

RESULTS

Three of the four compounds examined, 7-acetamido-nitrazepam, desmethyldiazepam and the chlordiazepoxide lactam, each exhibited two pKa values (Table 1) which correspond to protonation in acid and deprotonation in alkaline media. 7-Amino-nitrazepam gave three pKa values corresponding to two protonations in acid and one deprotonation in alkaline media.

Spectral data for each metabolite are given in Table 1, H_3A^{++}, H_2A^{+}, HA and A^{-} representing doubly protonated, protonated, neutral and deprotonated species respectively. The ultraviolet spectra corresponding to the four different absorbing forms of 7-amino-nitrazepam in the pH range 0–14 are shown in Fig. 1.

All the benzodiazepines (Clifford & Smyth, 1973) and their metabolites included in this study are polarographically reducible at the dropping mercury electrode, giving rise to one, two or three reduction waves at pH values relevant to solvent extraction procedures (Table 2). Linearity between wave height and concentration was generally observable in the range 10^{-4}—10^{-7}M. Above 10^{-4}M, adsorption effects limited the heights of the waves.

Having established polarography as a useful tool in monitoring the degree to which various benzodiazepines are extracted, we performed a variety of solvent extractions

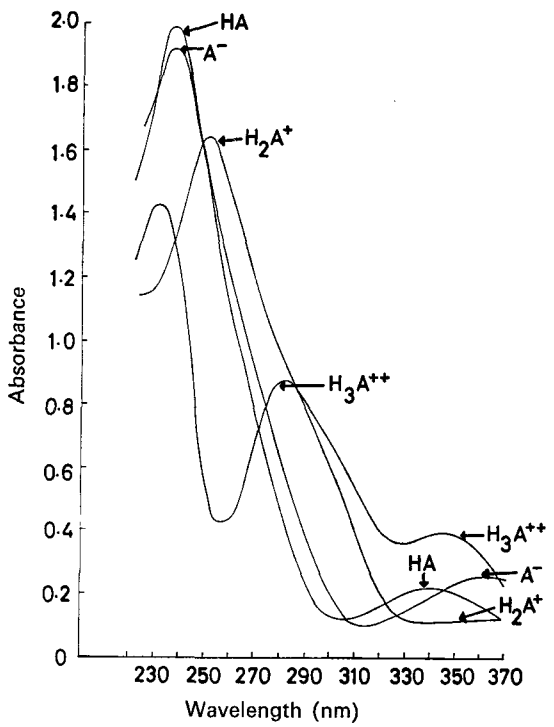


FIG. 1. The four absorbing forms of 7-amino-nitrazepam existing in the pH range 0–14.

Table 1. *pKa values and comparison of different spectral forms of four 1,4-benzodiazepine metabolites* (electronic spectra).

Compound	pK_1	pK_2	H_3A^{++} λmax	H_3A^{++} E(×10⁴)	H_2A^+ λmax	H_2A^+ E(×10⁴)	HA λmax	HA E(×10⁴)	A^- λmax	A^- E(×10⁴)
7-Acetamido-nitrazepam	3·2	12·4	—	—	256 290 (s) 330	1·8 0·8 0·1	245 265 (s) 330 (s)	1·6 1·4 0·1	245 (s) 262 (s) 280 (s) 355	1·5 1·4 0·8 0·2
7-Amino-nitrazepam	2·5* 4·6	13·1	233 281 303 (s) 345	2·5 1·6 1·2 0·7	253 285 (s)	2·9 1·6	240 267 (s) 340	3·5 1·6 0·4	238 270 (s) 361	3·4 1·6 0·5
Desmethyl-diazepam	3·5	12·0	—	—	235 283 363	2·0 0·8 0·2	230 250 (s) 313	2·3 1·1 0·2	233 258 (s) 275 (s) 342	2·4 1·5 0·8 0·4
Chlordiazepoxide lactam	4–5	11·5	—	—	237 305	3·6 1·1	237 305	3·6 1·1	242 255 (s) 304	3·4 3·3 0·7

* This value refers to a second protonation in acid media.

Table 2. *Polarographic reduction waves of nitrazepam, diazepam, chlordiazepoxide and some of their metabolites.**

Compound	Ep (V. v S.C.E.)	Relative heights	Supporting electrolyte
Nitrazepam**	−0·27, −0·84	4:4	BR buffer, pH 4
	−0·40, −1·02	4:3	BR buffer, pH 7
7-Acetamido-nitrazepam**	−0·83	2	BR buffer, pH 4
	−1·00	2	BR buffer, pH 7
7-Amino-nitrazepam**	−0·83	2	BR buffer, pH 4
	−1·02	2	BR buffer, pH 7
Diazepam	−1·22	2	M NaOH
Desmethyl-diazepam	−1·32, −1·65	1:1	M NaOH
Chlordiazepoxide	−0·76, −0·93, −1·36	2:2:4	BR buffer, pH 7
Chlordiazepoxide lactam	−0·69, −0·97	2:2	BR buffer, pH 7

* Concentration 5×10^{-5}M in supporting electrolytes containing 5% methanol.

** Concentration 10^{-6}M.

Table 3. *Solvent extractions of nitrazepam, diazepam, chlordiazepoxide, and some of their metabolites.*

Compound	Solvent	pH of aqueous phase	% Extracted from aqueous phase**
Nitrazepam*	Ethyl acetate	7	95–100
7-Acetamido-nitrazepam*	Ethyl acetate	7	90
	Dichloroethane	4	70
7-Amino-nitrazepam*	Ethyl acetate	7	95–100
	Dichloroethane	4	80
Diazepam	Chloroform	14	95–100
	Light petroleum	14	95–100
Desmethyl-diazepam	Chloroform	14	95
	Light petroleum	14	0–5
Chlordiazepoxide	Chloroform	7	95–100
Chlordiazepoxide lactam	Chloroform	7	95–100

* Concentration 10^{-6}M in aqueous phase

** Calculated by polarographic examination of aqueous phase before and after extraction.

at pH values selected from the pKa data. Table 3 summarizes these extraction results. Based on these findings, the following solvent extractions of mixtures of parent compound and metabolite(s) were carried out. A mixture, 10^{-6}M in nitrazepam and each of its two metabolites, was buffered with BR buffer at pH 7 and extracted with ethyl acetate for 20 min. The ethyl acetate layer was separated, the solvent evaporated and the residue dissolved in 5 ml of pH 7 BR buffer, before polarography was carried out. The resulting polarogram showed two waves at −0·4 and −1·02V with a ratio of wave heights of 1 : 1·7, similar to a standard solution of the three (each 10^{-6}M in pH 7 buffer) that had not been subjected to solvent extraction. A mixture, 5×10^{-5}M in diazepam and its desmethyl metabolite in 1M sodium hydroxide, was extracted with light petroleum (b.p. 40—60°) for 5 min. Polarography of the aqueous layer gave two waves at −1·32 and −1·65V with heights and peak potentials identical to a standard, 5×10^{-5}M desmethyl-diazepam in 1M sodium hydroxide containing 5% methanol. After evaporating off the solvent from the organic layer and dissolution of the residue in 1M sodium hydroxide containing 5% methanol, polarography showed one wave at −1·2V with height and peak potential identical to a standard 5×10^{-5}M diazepam in 1M sodium hydroxide containing 5% methanol. Finally, chlordiazepoxide and its lactam were extracted into chloroform by shaking a mixture of the two (5×10^{-5}M in each buffered at pH 7) with this solvent for 10 min. Polarography of the residue dissolved in BR buffer, pH 7, containing 5% methanol gave rise to the wave pattern illustrated in Fig. 2 (p. 16).

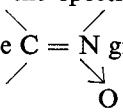
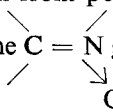
DISCUSSION

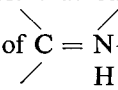
Spectra and sites of protonation and deprotonation

The spectrum of desmethyl-diazepam at a pH where it exists as a neutral molecule is practically identical to that of diazepam (Barrett & others, 1973), indicating very little effect on the spectrum by substituting a hydrogen atom for the methyl group in position 1.

On passing through the pK_1 value, both major bands shift to the red suggesting protonation at position 4 since modification of the $\diagdown\!\!\diagup C = N \diagup$ group should affect both bands. In alkaline media, the main spectral change involves band II moving from 250 nm to 258 nm. This small red shift could perhaps be attributed to deprotonation from position 1 but more likely from position 3 or 2 (formed by enolization of the 2,3-group).

The conformation of the diazepine ring in chlordiazepoxide is considerably different from that in the lactam as the half wave potentials corresponding to the 2 step reduction of the $\diagdown\!\!\diagup C = N \diagup\!\!\diagdown$ group are noticeably different for the two compounds (Table 2). Correspondingly, the spectrum of chlordiazepoxide (Barrett & others, 1973) shows, on protonation of the molecule, a blue shift of band I suggesting that the $\diagdown\!\!\diagup C = N \diagup\!\!\diagdown$ group is suitably aligned for interaction with the monosubstituted ring rather than

the trisubstituted one. On the other hand, the spectrum of the lactam shows no change on protonation, in agreement with the  group being aligned to neither ring. It is for this reason that band I in chlordiazepoxide occurs at a longer wavelength (260 nm) than in the lactam (237 nm). Deprotonation at position 1 would be expected to cause a red shift of band II which clearly does not happen. It is therefore suggested that deprotonation from position 3 or 2 (enol form) occurs and thereby increases the interaction of the  group with the phenyl ring in postion 5.

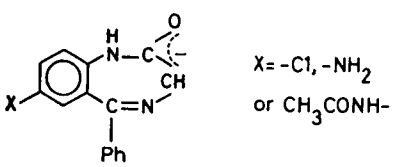
Protonation of the amine group in 7-amino-nitrazepam would be expected to cause a blue shift in band II. This occurs in going from the species H_2A^+ to H_3A^{++} where both bands undergo blue shifts. It is noticeable that band I undergoes a larger blue shift (20 nm) suggesting greater interaction of  with the trisubstituted ring than with the monosubstituted one. It is concluded that the lower pKa value corresponds to protonation on the amine function and that the first protonation on the neutral molecule occurs at position 4 as is found with other benzodiazepines (Barrett & others, 1973). A small spectral change occurs in alkaline media not consistent with removal of a proton from position 1 (a large red shift would be expected in this case) but more likely corresponding to removal of a proton from position 3 or 2 (enol form).

Protonation in acid media of 7-acetamido-nitrazepam results in a shift of both major bands to the red consistent with protonation in position 4 since protonation at position 1 or on the nitrogen of the 7-acetamido substituent should cause a considerable blue shift of band II. As with 7-amino-nitrazepam, little spectral change is noticed in passing through the pK_2 value suggesting loss of a proton from position 3 or 2 (enol form).

A more quantitative interpretation of the spectra of the benzodiazepines may be achieved by computer resolution and work is in progress to this end (Barrett & Bolt, unpublished).

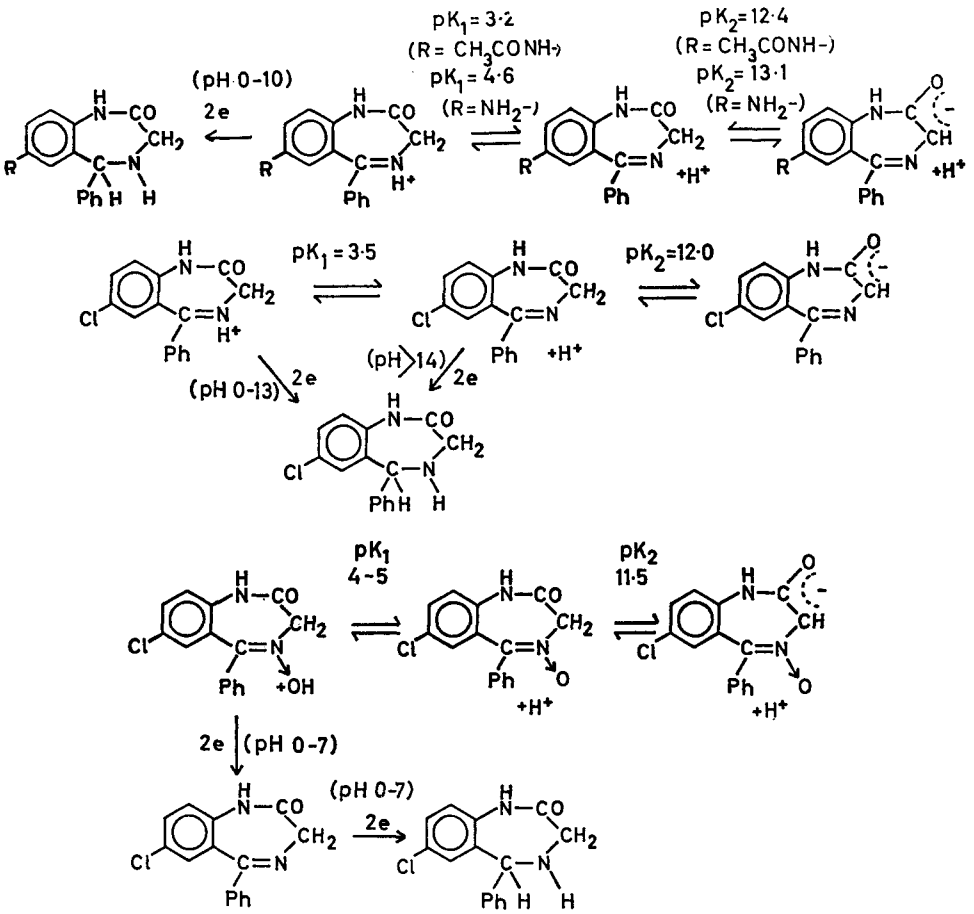
Explanation of observed pKa values

Protonations in acid media (except those corresponding to the amino group of 7-amino-nitrazepam) of desmethyl-diazepam, 7-amino-nitrazepam and 7-acetamido-nitrazepam are all within the range expected for the C = N group (Barrett & others, 1973). Deprotonations in alkaline media all occur at high pKa values (11·5 to 13·1) consistent with the difficulty of formation of anions of type:



Polarography

Recently Clifford & Smyth (1973) showed in polarographic investigations on aqueous solutions of the 1,4-benzodiazepines that at least three species were present, the monoprotonated, neutral and anionic forms, in agreement with the spectral findings (Barrett & others, 1973). The protonated form was found generally polarographically reducible over a wide pH range and the resulting waves were suitable for analytical measurements at the milligram level they occur in formulations or at the nanogram level after extraction from the body fluids. The same can be said about the polarographic behaviour of the four metabolites, which is schematically collected below along with the spectral data. The pH ranges in brackets are those where the depicted species is polarographically reduced.



Solvent extractions

In an attempt to produce a selective extraction of 7-acetamido-nitrazepam from 7-amino-nitrazepam, the distribution of the individual compounds was investigated between dichloroethane and BR buffer pH 4 (Table 3), a pH at which the former compound predominately exists as a neutral molecule and the latter is monoproton-

ated. Unfortunately, both were extracted (70–80%) presumably due to ion pair formation in the case of the amino compound. However, an extraction of a mixture of nitrazepam and its two metabolites into ethyl acetate at pH 7 where they all exist as neutral molecules was successful in producing a polarographic method of differentiation of the parent compound from total metabolites. The first of the two waves observed after evaporation of the solvent and dissolution of the residue in BR buffer pH 7 corresponds solely to the 4e reduction of $-NO_2$ to $-NHOH$ in the parent compound, whereas the second corresponds to the 1e reduction * of the product of the first reduction, $-NHOH_2^+$ to $-NH_2$ and the sum of the 2e azomethine reductions occuring on all three compounds. From a computation of these data, the ratio of the wave heights after extraction of an equimolar mixture of the three should be 1:1·75, in agreement with the experimental value. In practice, therefore, the concentration of nitrazepam can be found by referring the height of the first wave to a predetermined calibration plot for a nitrazepam $-NO_2$ group reduction in BR buffer, pH 7. The total metabolites concentration is found by subtracting three quarters the height of the first wave from that of the second and referring this height to a calibration plot for a nitrazepam metabolite $\diagdown C = N \diagup$ group reduction in BR buffer, pH 7.

Chloroform was found to extract both diazepam and desmethyl-diazepam at pH 14 where the former exists as a neutral molecule and the latter as an anion.

However, the less polar solvent, light petroleum, was found to differentiate a mixture of these two species after their extraction from N sodium hydroxide as indicated in the results.

Polarographic differentiation could still be used after extraction of both species at pH 7 (where both exist as neutral molecules) and eventual dissolution in N sodium hydroxide, a medium in which both species have a different polarographic behaviour (Table 2).

This difference in behaviour is presumably due to the polarographic manifestation of an acid base equilibrium in the case of desmethyl-diazepam which splits the single

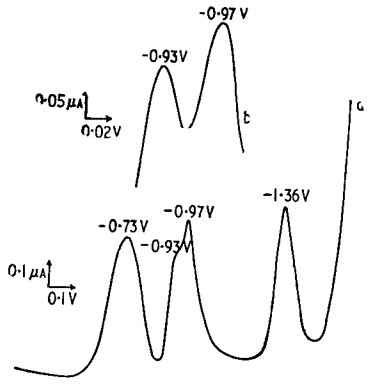


FIG. 2. Differential pulse polarograms of a mixture of chlordiazepoxide and its lactam (each 5×10^{-5} M) co-extracted by $CHCl_3$ from an aqueous mixture of the two, buffered at pH7 (a) starting potential −0·3 V, scanning rate 10 mV s^{-1}, (b) starting potential −0·91 V, scanning rate 2 mV s^{-1}.

* The wave height is equivalent to a one electron reduction since the polarographic pK value for the hydroxylamine function is 7·0 and only the protonated form is reducible at the dropping mercury electrode (Clifford & Smyth, 1973).

$\backslash C = N /$ reduction wave into two waves, the one at the more negative potential corresponding to reduction of the conjugate base.

Since chlordiazepoxide is very susceptible to rearrangement in alkaline media, no attempt was made to differentiate the anionic form of the lactam from the neutral form of the parent compound by a selective solvent extraction in N sodium hydroxide. Instead, both were extracted as neutral molecules from BR buffer, pH 7, into chloroform.

Polarography of the residue in pH 7 buffer after evaporation of the solvent yielded a three wave pattern (Fig. 2), the first wave being due to the amalgamation of *N*-oxide reductions occurring in both the parent compound and the lactam. The second wave occurred at −0·97 V with a shoulder at −0·93 V which could be resolved into two well-defined peaks using a slow rate of scanning (2 mV s^{-1}). These two reduction waves correspond to the 2e reductions of the $\backslash C = N /$ groups in lactam and parent compound respectively. The third wave is due to the 4e reduction of the $N = C(NHCH_3)$ group in the parent compound, resulting in reductive splitting of the carbon-methylamino bond (Clifford & Smyth, 1973).

In practice, this results in simultaneous measurement of the metabolite (at −0·97 V) and parent compound (at −1·36 V) after reference to predetermined i_{lim}-concentration curves for the reduction of the $\backslash C = N /$ group in the lactam and the $N = C(NHCH_3)$ group in chlordiazepoxide respectively (both at pH 7).

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