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# A SPECTROMETRIC, SEPARATION AND VOLTAMMETRIC STUDY OF THE COMPLEXATION REACTIONS OF BROMAZEPAM WITH IRON(II), COPPER(II) AND COBALT(II)

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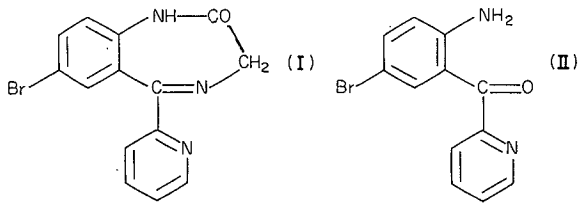
SUMMARY

Bromazepam, in the form of a cationic iron(II) chelate, can be determined spectrophotometrically at 588 nm with a limit of detection of ca. $10^{-6}$ M. When this chelate is ion-paired with perchlorate, it can be extracted into organic solvents such as 1,2-dichloroethane and 4-methyl-2-pentanone, and determined by atomic absorption spectrometry with a limit of detection of $1.5 \times 10^{-5}$ M bromazepam at the iron resonance 248.3-nm line. Ion-pairs involving the Fe(II), Cu(II) and Co(II) chelates and perchlorate can be separated by h.p.l.c. using a $C_{18}$ reverse-phase column and a mobile phase of 4:1 water—methanol, with a u.v. detector at 242 nm. This approach allowed for the determination of iron(II) ions in aqueous solution with a limit of detection of $10^{-8}$ M. The h.p.l.c. method could also be used to quantify bromazepam spiked in plasma in the concentration range 1—10 $\mu$g ml$^{-1}$, following extraction of bromazepam from plasma and subsequent formation of the iron(II) ion-pair. Copper(II) forms a labile chelate with bromazepam in pH 4.8 acetate buffer which, when subjected to differential pulse voltammetry at the hanging mercury drop electrode, gives rise to a catalytic phenomenon which can be utilised for the determination of bromazepam in the concentration range $10^{-5}$—$10^{-9}$ M.

Bromazepam [I; 7-bromo-1,3-dihydro-5-(2-pyridyl)-2*H*-1,4-benzodiazepin-2-one] has aroused clinical interest as an anti-anxiety agent [1—3]. Its metabolic rate has been investigated [4—6] and it has been found that only the parent compound is present in blood after therapeutic administration. Both gas—liquid chromatographic methods with electron capture detection [6, 7] and differential cathode-ray polarographic methods [8] exist for the determination of bromazepam in plasma with detection limits of 5—10 ng ml$^{-1}$ of plasma [6, 7] and 50 ng ml$^{-1}$ [8]. Smyth et al. [9] have made a spectral, polarographic and voltammetric study of bromazepam and produced an indirect analytical method for the determination of $10^{-7}$ M concentrations of bromazepam, using differential pulse anodic stripping voltammetry of the free copper(II) ions remaining after complexation of an excess of this metal ion with bromazepam.

The existence of the $\alpha,\alpha'$-dipyridyl moiety enables bromazepam to form complexes with divalent metal ions such as copper(II) and cobalt(II) [9]. This behaviour has been studied spectrophotometrically in relation to iron(II) ions [10] and applied to the determination of iron(II) [11] and haemoglobin [12] in serum.




This paper is concerned with a study of the chelation of bromazepam (I) with Fe(II), Cu(II) and Co(II) using spectroscopic techniques such as u.v. and visible spectrophotometry and atomic absorption spectrometry, separation techniques such as solvent extraction and ion-pair high-performance liquid chromatography (h.p.l.c.) and differential pulse voltammetry, at the hanging mercury drop electrode with a view to enhancing the sensitivity and selectivity of analytical methods for the determination of trace concentration of bromazepam in the presence of non-chelating molecules such as benzophenone metabolites (II) and 1,4-benzodiazepines without pyridine groups, i.e., all other commercially available 1,4-benzodiazepines. The h.p.l.c. separation of bromazepam and its Cu(II), Fe(II) or Co(II) chelates, quite apart from its use in the determination of down to $10^{-8}$ M concentrations of iron(II) ions, could be of value in biochemical investigations on the binding of this benzodiazepine drug to trace concentrations of relevant metal ions contained in body fluids.

## EXPERIMENTAL

### *Preparation of complexes*

A stock solution ($1 \times 10^{-3}$ M) of the iron (bromazepam)$_3^{2+}$ chelate was prepared by dissolving 0.09 g of bromazepam (in 5 ml of methanol; Hoffmann-La Roche, Nutley, NJ) and 0.03 g of iron(II) sulphate in 100 ml of deionised water. This solution was subsequently diluted with deionised water to yield solutions covering the concentration range $1 \times 10^{-6}$—$10^{-3}$ M in the iron chelate. The copper(II) and cobalt(II) complexes were prepared similarly. All were purple in colour.

### *Procedures*

*U.v.—visible spectrophotometry.* The spectra of solutions in the above concentration range with respect to the iron(II) chelate were scanned in the region 200—700 nm using an SP5000 spectrophotometer, and a graph of absorbance vs. concentration was constructed for the 588-nm band. It should be noted that the absorbance of the ligand at 242 nm is considerably

enhanced by chelation, so that u.v.—visible detection following h.p.l.c. separation could be attempted at both wavelengths.

*Solvent extraction of the iron(II) chelate and u.v.—visible spectrophotometry.* Equal volumes (10 ml) of aqueous standard solutions of the iron chelate ($10^{-3}$—$10^{-6}$M) and 1,2-dichloroethane or 4-methyl-2-pentanone were equilibrated for 5 min, and the concentration of the complex remaining in the aqueous phase was estimated by scanning from 370 nm to 700 nm. This was repeated with other solutions saturated with potassium perchlorate.

*Solvent extraction of the iron(II) chelate and atomic absorption spectrometry.* A range of solutions containing various concentrations of bromazepam (0.1—1.0 × $10^{-4}$ M) in water/methanol was prepared from a $10^{-3}$ M stock solution. Solutions of 1 × $10^{-3}$ M iron(II) sulphate and saturated potassium perchlorate were also prepared in deionised water. Aliquots (10 ml) of the various bromazepam solutions were placed in separating funnels along with 10 ml of the iron(II) solution and either 10 ml of deionised water or 10 ml of the saturated perchlorate solution. These mixtures were equilibrated with 30 ml of the organic solvent for 5 min, and the organic layers were drawn off. Care was taken to ensure minimal loss of the solvent by evaporation and a non-bromazepam blank extraction was also done. The iron content of the organic layer was determined by using the resonance line at 248.3 nm and an oxygen—acetylene flame. Graphs of absorbance at 248.3 nm vs. the concentration of the complex were then constructed.

*H.p.l.c. separation of the ion-pairs of the Cu(II), Co(II), and Fe(II) chelates with perchlorate.* The complexes were prepared as indicated under Preparation of complexes. Potassium perchlorate (1 M) was added to the $10^{-3}$ M stock solutions of the complexes to form the appropriate ion-pairs prior to dilution of the solutions to yield $10^{-4}$—$10^{-6}$ M concentrations of the ion-pairs. For dilutions, the water/methanol (4:1) mobile phase was used, to avoid the interfering peaks from solvent front effects which occurred on injection of purely aqueous samples.

The reverse-phase $C_{18}$ column LiChrosorb (25 cm × 4.6-mm i.d.; 10-$\mu$m particles) was chosen for the h.p.l.c. investigation of the Fe(II), Cu(II) and Co(II) ion-pairs. The flow rate was (1 ± 0.01) ml $min^{-1}$ at a piston pressure of 40 Bar. A u.v.—visible detector was operated at 242 nm and 588 nm.

*Differential pulse voltammetry of the copper(II) chelate at the hanging mercury drop electrode.* A PAR Model 174A polarographic analyzer was used in conjunction with a Model 303 static mercury drop electrode. The drop time was set at 0.5 s in the differential pulse mode. The concentration of copper(II) ions was kept constant at $10^{-4}$ M while that of bromazepam was varied from $10^{-9}$ to $10^{-5}$ M. A pre-electrolysis time of 3 min at an initial potential of 0 V was used, together with a scan rate of 5 mV $s^{-1}$ and a modulation amplitude of 25 mV.

## RESULTS AND DISCUSSION

*Solvent extraction of the iron(II) chelate and u.v.—visible spectrophotometry*

Bromazepam (I), in the form of a cationic iron(II) chelate, gives a linear calibration curve of absorbance at 588 nm vs. concentration in the range $10^{-3}$—$10^{-6}$ M of the complex. This calibration curve was then used in order to monitor the transfer of this purple complex from the aqueous phase (pH 7.0) to 1,2-dichloroethane or 4-methyl-2-pentanone. Visual observation showed that transfer was only possible in the presence of perchlorate as an ion-pairing agent for the iron(II) chelate, $[Fe(bromazepam)_3]^{2+}$. When the visible spectrum of the aqueous phase was run after an extraction, it was found that its shape and the position of the absorption bands changed somewhat, invalidating the use of the calibration curve even when the organic solvents were used to presaturate the aqueous phases prior to preparation of the stock solutions and extraction. After extraction, with perchlorate present, the visible spectrum again changed.

*Solvent extraction of the iron(II) chelate and atomic absorption spectrometry*

To overcome the afore-mentioned effect, it was decided to monitor the transfer of the ion-pair to the organic solvent by measuring the atomic absorption of the iron(II) in the organic phase. It would also have been possible to monitor the decrease in the iron(II) concentration in the aqueous phase after extraction, but because iron(II) was added in excess over bromazepam for these extractions, the results would not have been accurate.

Linear calibration plots of the atomic absorption of the organic phase at the iron resonance 248.3-nm line vs. concentration of the complex in the aqueous phase were obtained for both 1,2-dichloroethane and 4-methyl-2-pentanone in the concentration range 0.05—1.00 $\times$ $10^{-4}$ M. This lower level (which corresponds to a limit of detection of 1.5 $\times$ $10^{-5}$ M in bromazepam) was established by the extraction of non-bromazepam-containing neutral compounds of the excess of ion(II) not involved in the chelating process (iron(II) perchlorate or sulphate).

*H.p.l.c. separation of the ion-pairs of the Cu(II), Co(II) and Fe(II) chelates with perchlorate and application to the determination of iron(II) and bromazepam*

Tests on the h.p.l.c. behaviour of these ion-pairs on a 10-$\mu$m LiChrosorb $C_{18}$ reverse-phase column, with a 4:1 water/methanol mobile phase and u.v.—visible detection at 242 nm, showed that the retention times for bromazepam, $Fe(bromazepam)_3(ClO_4)_2$, $Cu(bromazepam)_3(ClO_4)_2$, and $Co(bromazepam)_3(ClO_4)_2$ were 296, 324, 365, and 339 s, respectively. The complexes were prepared with excess of bromazepam, which also appeared on the chromatograms at 296 s.

Of the three ion-pairs, only iron(II) yielded linear calibration plots of peak area vs. concentration of the ion-pair. Reproducibility of the absorbances of

the copper and cobalt ion-pairs was difficult to achieve, because of chelate lability and consequent partial breakdown of the chelates on the stainless steel column. This was illustrated when the Fe(II), Cu(II) and Co(II) ion-pairs were prepared in excess of metal ion and subjected to h.p.l.c. with u.v. detection at 242 nm. The iron(II) ion-pair gave a single peak at 324 s whereas the copper and cobalt ion-pairs both gave sizable peaks at 296 s corresponding to free bromazepam. To ensure that the iron(II) ion-pair was in fact that which was being observed, this ion-pair was injected onto the column with the detector set at 588 nm, a wavelength specific for the $Fe(bromazepam)_3$ species. A single peak was observed at 320 s which corresponds to $Fe(bromazepam)_3(ClO_4)_2$. No peak was observed for bromazepam because it does not absorb at this wavelength.

When 20-$\mu$l injections and a helium-degassed mobile phase were used, a linear calibration plot of peak area vs. concentration of the iron(II) ion-pair was obtained in the concentration range $10^{-3}$—$10^{-7}$ M. The relative standard deviations of the peak areas for repeated injections of this ion-pair at concentrations of $10^{-3}$ M and $10^{-7}$ M varied from 0.07% to 0.70%, respectively. The signal-to-noise ratio was found to be 2:1 on injection of a $10^{-8}$ M solution of the iron(II) ion-pair; this concentration was then designated as the limit of detection for this ion-pair under the h.p.l.c. conditions described above. The number of theoretical plates was 20 736, the height equivalent of the theoretical plate was $1.2 \times 10^{-3}$ cm and the resolution factor $R$ was 3.1 for the separation of the iron(II) ion-pair and the excess of bromazepam.

The h.p.l.c. procedure was briefly evaluated for the determination of bromazepam as the iron(II) ion-pair after extraction of bromazepam spiked in human plasma. Proteins were precipitated by the addition of 3:1 $Na_2CO_3$/$NaHCO_3$ to 1 ml of the spiked plasma sample followed by vortex mixing and separation of the supernatant liquid. Chloroform was used to extract bromazepam from the supernatant liquid, the chloroform was evaporated off at 50°C on a water bath, and the residue was reconstituted in the usual aqueous methanol mobile phase. Examination of this solution by h.p.l.c. showed that recoveries of the bromazepam were about 80% and that the bromazepam peak was well separated from other u.v.-absorbing substances co-extracted from the plasma. A ten-fold excess of iron(II) was then added to the above-mentioned residue after reconstitution in the aqueous methanol mobile phase; the ion-pair with perchlorate was then extracted and subjected to h.p.l.c. as previously described. The chromatograms showed a peak corresponding to free bromazepam as well as the iron(II) ion-pair, showing that not all the bromazepam was chelated under the conditions used. Even when a further excess of iron(II) was added to the above solution prior to injection, the height of the peak corresponding to the iron(II) ion-pair did not alter significantly. However, when the solution conditions following extraction and reconstitution were changed, i.e., when the pH of the reconstituted extract (9.2) was altered to the optimum pH of ca. 7.0 for chelation by the addition of anhydrous acetic acid, and the iron(II) chelate and perchlorate

ion-pair were then formed, no free bromazepam was observed in the chromatogram. A linear calibration graph of peak area corresponding to the iron(II) ion-pair versus the concentration of bromazepam spiked in the plasma was found in the concentration range 1—10 $\mu g\,ml^{-1}$. Detection of 0.1 $\mu g\,ml^{-1}$ concentrations of bromazepam spiked in plasma could also be accomplished by this method.

*Differential pulse voltammetry of the copper(II) chelate at the hanging mercury drop electrode*

In the presence of excess of copper(II) ions, it was found that the height of the bromazepam reduction wave corresponding to the >C=N— group at —0.57 V (vs. s.c.e.) in 0.1 M acetate buffer (pH 4.8) was enhanced after the hanging mercury drop electrode had been conditioned at 0 V for 3 min. This applied to solutions which were $10^{-9}$—$10^{-5}$ M in bromazepam and $10^{-4}$ M in copper(II) ions. Because bromazepam is not adsorbed at the h.m.d.e. under these conditions [the wave height from the >C=N— reduction of bromazepam does not increase with increasing conditioning/plating time at an applied potential of 0 V (vs. s.c.e.) in 0.1 M acetate buffer pH 4.8], a mechanism must operate whereby bromazepam is concentrated at the electrode surface by adsorption of the copper(II) chelate. The fact that the reduction potential corresponding to the enhanced peak is identical to the reduction potential of "free" bromazepam would suggest that at the time of charge transfer, the chelation of copper(II) to bromazepam is no longer effective at the electrode surface and that copper ions are involved in a catalytic mechanism. A further paper will be concerned with the mechanism of this phenomenon. A plot of peak height corresponding to the >C=N— reduction of bromazepam vs. the concentration of bromazepam was found to be linear in the concentration range $10^{-5}$—$10^{-9}$ M.

*Conclusion*

The copper(II) and iron(II) chelates with bromazepam could be used for selective determinations of trace concentrations of this 1,4-benzodiazepine by either differential pulse voltammetry at the h.m.d.e. or by spectrometric procedures after extraction or h.p.l.c. The h.p.l.c. method would be expected to be the most selective and sensitive for the determination of bromazepam in complex matrices such as plasma, especially when non-chelating degradation products/metabolites and other 1,4-benzodiazepines are present.

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