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Analysis of new serotonergic anxiolytics by liquid chromatography*

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Abstract: A simple isocratic procedure was developed for the analysis of new serotonergic anxiolytics and the related compounds in bulk materials, pharmaceutical formulations and in biological samples. The system may be applied for the assay of other serotonergic anxiolytics of related structure such as buspirone. The liquid chromatographic assay utilizes a reversed-phase C_{18} column, a mobile phase consisting of a mixture (55:45, v/v) of (A) buffer potassium dihydrogen phosphate (0.05 M) containing sodium lauryl sulphate (0.005 M) and (B) acetonitrile. A fluorescence detection is used with λ_{ex} 237 nm; λ_{em} 374 nm. The accuracy, precision and sensitivity of the proposed method are established. Standard curves are linear with respect to concentration in the range 0.05–7.5 μg ml^{-1}. The method also allows the separation and identification of related compounds at concentrations below 0.01%.

Keywords: *Zalospirone; gepirone; ipsapirone; related substances; ion-pair LC; fluorescence detection.*

Introduction

Zalospirone [1] (**Z**), 3*a*,4,4*a*,6*a*,7,7*a*,hexahydro-2-{4-[4-(2-pyrimidinyl)-1-piperazinyl]-butyl}-4,7-ethene-1H-cyclobut[f]isoindole-1,3(2H)-dione hydrochloride, gepirone [2] (**G**), 4,4′-dimethyl-1{4-[4-(2-pyrimidinyl)-1-piperazinyl]-butyl}-piperidine-2,6-dione hydrochloride, and ipsapirone [3] (**I**), 2-{4-[4-(2-pyrimidinyl)-1-piperazinyl]-butyl}-1,2-benzisothiazol-3(2H)-one-1,1-dioxide hydrochloride are non benzodiazepinic anxiolytic agents belonging to the new generation of serotonergic anxiolytics [4] such as the prototype buspirone. The common [(2-pyrimidinyl)-1-piperazinyl] butyl moiety (Fig. 1) confer to **Z**, **G** and **I** similar fluorescence spectra.

Only a few analytical procedures have been reported until now for this relatively new class of compounds. Two gas chromatographic quantitative methods have been proposed for the analysis of buspirone, the first uses flame ionization detection [5] and the second electron capture detection [6]. A capillary GC–MS method [7], an ion-pair HPLC [8] and a specific HPLC–DAD [9] technique for the **G** analysis also have been described.

In the present paper a direct reversed-phase ion-pair method for the analysis of **Z**, **G** and **I** and related compounds (Fig. 2) is proposed; a fluorimetric rather than an UV detector has been selected in order to improve the specificity and the sensitivity of the method. Higher sensitivity is helpful for specific assay at the therapeutic level of drugs in biological fluids and for the detection of impurities. The method can be applied to the analysis of raw materials, pharmaceutical formulations and biological samples.

Experimental

Apparatus

A high-performance liquid chromatograph model 501 (Waters, Milford, MA, USA) equipped with a LS5-B luminescence spectrometer (Perkin–Elmer, Norwalk, CT, USA) and an integrator model 745B (Waters, Milford, MA, USA) was used in the investigation. Standard and sample solutions were injected via a Rheodyne valve injector model 7125 (Berkeley, CA, USA) fitted with a 20 μl loop.

Chromatographic conditions

The chromatographic separation of **Z**, **G**, **I** and related compounds was achieved by means of a ODS Hypersil (Shandon, Runcorn, Cheshire, UK) column (150 × 4.6 mm i.d. particle size 5 μm). The eluent was 0.005 M

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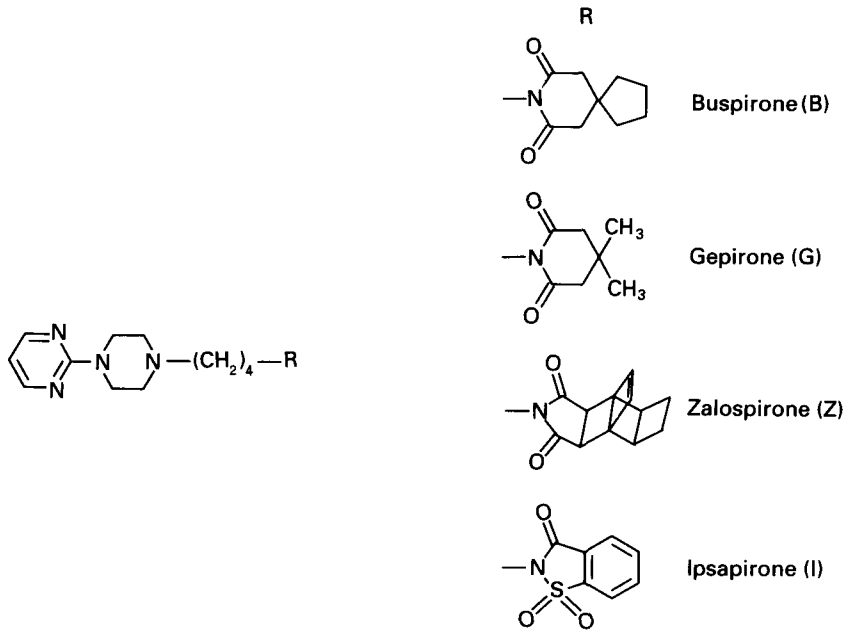

Figure 1
Chemical structures of buspirone, gepirone, zalospirone and ipsapirone.

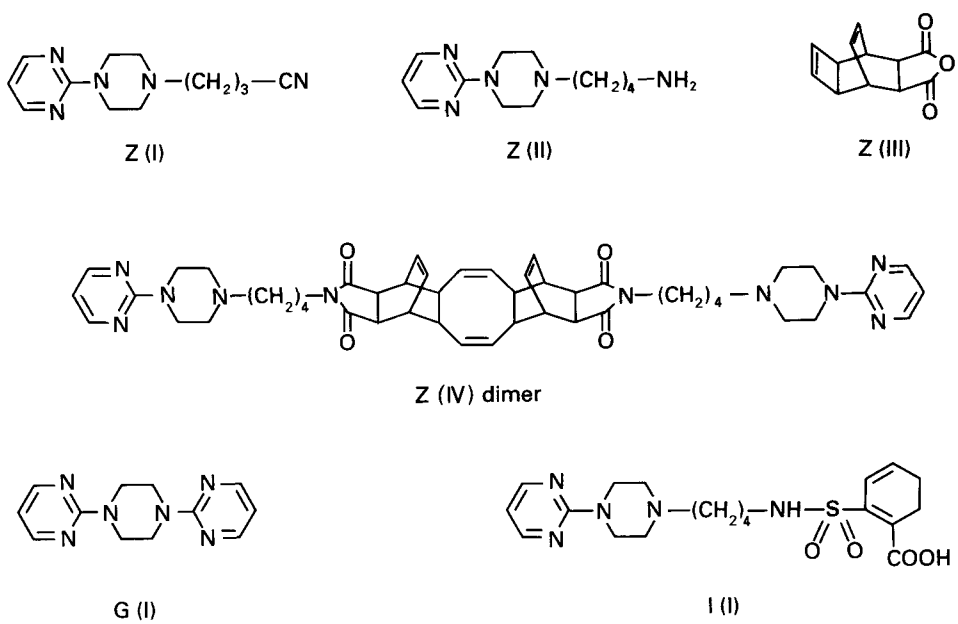

Figure 2
Chemical structures of the main impurities related to the synthesis procedure of zalospirone, gepirone and ipsapirone.

sodium lauryl sulphate in 0.05 M potassium dihydrogen phosphate buffer solution (pH 4) and acetonitrile (55:45, v/v) delivered at a flow rate of 1 ml min^{-1} at room temperature.

Chemicals and reagents

Zalospirone (Wyieth), gepirone (Bristol), ipsapirone (Bayer) and pure samples of impurities were generous gifts from their respective manufacturers. HPLC-grade acetonitrile and the other chemicals (all of analytical reagent grade) were obtained from Carlo Erba (Milan, Italy) and Merck (Darmstadt, Germany). Borate buffer pH 10 was obtained from Fluka AG (CH-9470 Buchs, Switzerland). The mobile phase was filtered through a 0.45 μm-pore filter (Alltech, Deerfield, IL, USA) and degassed *in vacuo* and in

an ultrasonic bath before use. Double distilled water was used throughout the study. Sep-Pak C_{18} cartridges were obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA).

Standard solutions

Stock solutions (1 mg ml^{-1}) were prepared by dissolving 10 mg of the respective compounds in 10 ml of water. The solutions were stable in the dark at 4°C for at least 2 months. Standard solutions containing 0.5 – 5 μg ml^{-1} were prepared by diluting the stock solution with the mobile phase just before use.

Qualitative analysis

The reference standard solutions of **Z**, **G**, **I** and the corresponding related compounds were analysed in order to optimize the chromatographic conditions and to obtain the fluorescence spectral data. The qualitative analysis of raw materials was carried out subsequently.

Sample preparation

Bulk drugs. An accurately weighed amount of **Z** or **G** or **I** was dissolved in water to obtain 1 mg ml^{-1} solutions.

Serum samples. To aliquots of 1 ml of rabbit plasma were added 10 and 100 μl volumes, respectively of the **Z**, **G** and **I** solutions containing 10 μg ml^{-1} of drug, and 200 μl of borate buffer (pH 10). The sample was then loaded on to an activated reversed-phase C_{18} cartridge. The cartridge was washed with water (5 ml) and then with water–methanol (50:50, v/v) (1 ml). Finally, the retained drug was eluted with methanol (5 ml). The eluate was evaporated to dryness under a nitrogen stream at room temperature. The resulting residue was dissolved in 1 ml of mobile phase and 20 μl was injected onto the HPLC column.

Results and Discussion

The chromatographic conditions of the proposed method enable the separation of **Z**, **G**, **I** and their fluorescent impurities. Figure 3 shows the fluorescence spectra of solutions of **Z**, **G** and **I** in the mobile phase and the chromatographic profile obtained with a mixture of the three drugs in solution. The products were identified by their retention times, spiking of the mixture successively with each standard drug and their fluorescence spectrum.

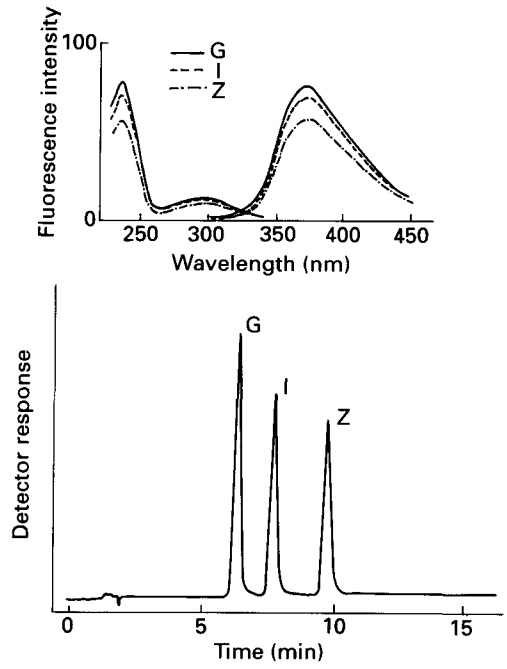

Figure 3
Fluorescence excitation and emission spectra of gepirone, ipsapirone and zalospirone solutions in the mobile phase. Typical chromatogram of a solution containing gepirone, ipsapirone and zalospirone working standards. Column: Hypersil C_{18}, 5 μ; mobile phase: (A) 0.005 M sodium lauryl sulphate in 0.05 M buffer potassium dihydrogen phosphate solution (pH 4) and (B) acetonitrile (55:45, v/v) at a flow rate of 1 ml min^{-1}; fluorescence detection at λ_{ex} 237 nm and λ_{em} 374 nm.

In order to verify the utility of the method for the quality control of **Z**, **G** and **I** three different working standard mixtures, each containing one drug and its potential impurities were prepared. Figure 4 shows that each drug was well separated from the corresponding impurities. Under the same conditions the **Z**(III) impurity of **Z** could not be detected being a non-fluorescent compound (this impurity was determined by a TLC procedure). The experimental conditions enabled the determination of **Z**(IV) dimer impurity in its *cis*- and *trans*-forms [Fig. 4(a)]; a retention time of about 20 min can be obtained with a flow rate of 1.8 ml min^{-1} [Fig. 4(b)]. For the detection of **G**(I) the following conditions were used: λ_{ex} 318 nm; λ_{em} 413 nm.

The linearity of detector response versus amount of injected standard compounds was checked by the injection of four different solutions. A linear relationship between concentration and fluorescence signal was

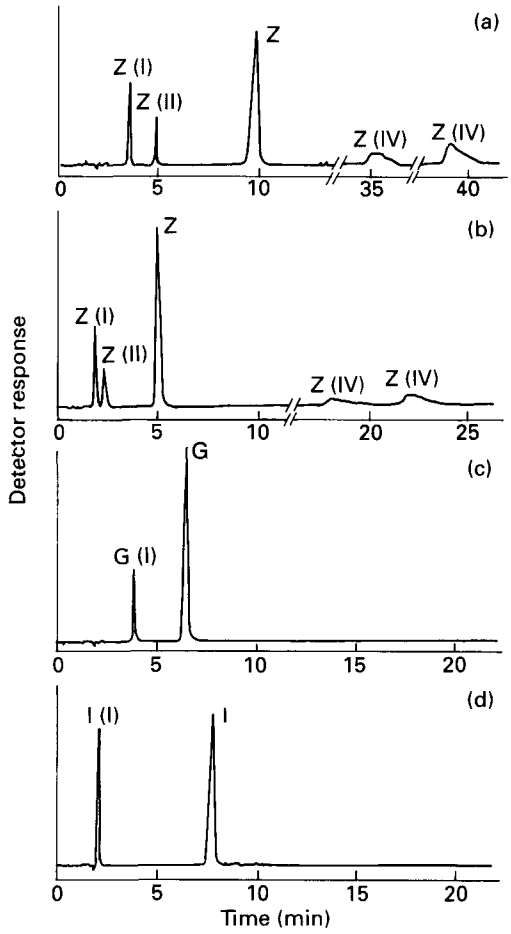

Figure 4
Chromatographic separation of the main compound and related impurities (see Fig. 2) of zalospirone (a and b), gepirone (c) and ipsapirone (d). Chromatographic conditions as in Fig. 3 with the following exceptions: (b) flow rate 1.8 ml min^{-1} and (c) λ_{ex} 318 nm and λ_{em} 413 nm.

observed in the range 0.05–7.5 μg ml^{-1}. The correlation coefficient for **Z**, **G** and **I** was found to be 0.9983, 0.9989 and 0.9998, respectively. The precision of the proposed HPLC procedure was confirmed by running 10 replicate samples, each containing 1.5 μg ml^{-1} of the drug in the final solution. At this concentration the relative standard deviations (RSD) for **Z**, **G** and **I** were 0.8, 0.6 and 1.1, respectively, indicating good precision and reproducibility. The detection limit at a signal-to-noise ratio of 1:3 was about 0.15 ng for each compound, with the exception of **G**(I) and **Z**(IV) where the detection limits were 10 and 3 ng, respectively.

The described method has been applied to the quality control of bulk materials and in the analysis of the biological samples.

The chromatographic profile of **G** bulk material did not reveal the presence of **G**(I); however two peaks of non-identified impurities were present, the relative retention times were about 0.40 and 0.47 and relative areas were about 0.2 and 1%. The chromatographic profile of **I** bulk material shows the presence of **I**(I) at very low concentration level (<0.05%) and that of **Z** showed the presence of **Z**(I) and **Z**(II) at trace levels (<0.01%), while the dimer was not observed. To identify **Z**(III), a non-fluorescent impurity, a TLC assay was performed by using silica gel plates with a mobile phase consisting of $CHCl_3$–methanol–ammonia (90:9:1, v/v/v). R_f [**Z**(III)]: 0.22; R_f [**Z**]: 0.90. This impurity was not present in **Z** raw material.

In Fig. 5 the chromatogram of a pre-treated blank serum and those of **G**, **Z** and **I** in spiked serum sample are presented (see Experimental). No interferences from the fluorescent endogenous compounds are

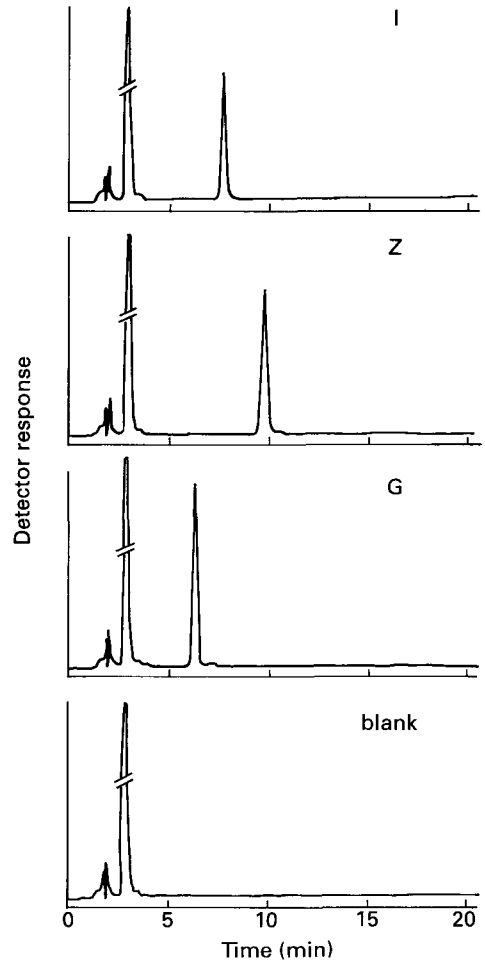

Figure 5
Chromatograms of a blank serum and of serum samples spiked with gepirone, zalospirone or ipsapirone. Chromatographic conditions as in Fig. 3.

observed. The recovery of **G** or **Z** or **I** from serum samples spiked with two different concentrations of drug was determined after prepurification by comparing the peak areas with those of standard solutions. The mean recoveries obtained for **Z**, **G** and **I** are reported in Table 1.

Table 1
Recoveries of **G**, **Z** and **I** from plasma (n = 3)

Compound	Amount added (ng ml^{-1})	Mean recovery (%)
Gepirone.HCl	100	98.4
	1000	97.8
Ipsapirone.HCl	100	98.4
	1000	94.2
Zalospirone.HCl	100	96.1
	1000	97.7

Conclusions

The method described here provides a sensitive and specific assay for **Z**, **G** and **I** and corresponding related compounds either as bulk materials or in biological fluids. It is shown to have the requisite accuracy and precision for routine use in the laboratory.

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