ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
20 (1999) 357–362

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Flow-injection fluorimetric determination of 1,4-benzodiazepines in pharmaceutical formulations after acid hydrolysis

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Received 29 June 1998; received in revised form 17 December 1998; accepted 1 January 1999

Abstract

A simple, rapid and fully automated flow injection method with fluorimetric detection after hydrolysis with H_2SO_4 in ethanolic or methanolic medium at room temperature has been developed for the determination of 1,4-benzodiazepines (oxazepam, diazepam and nitrazepam) in pharmaceutical formulations. The calibration curves are linear in the ranges (mg ml^{-1}) of oxazepam (0.025–0.150), diazepam (0.010–0.125) and nitrazepam (0.010–0.150), with detection limits of 0.01, 0.005 and 0.005 mg ml^{-1}, respectively, and RSD (1% ($n = 10$). The measurement throughput is 60 h^{-1} using a 200-μl sample volume obtained by the direct dissolution of formulations in alcohol.

Keywords: Fluorimetry; Flow injection; Oxazepam; Diazepam; Nitrazepam; 1,4-benzodiazepines; Pharmaceutical analysis

1. Introduction

1,4-Benzodiazepines are very interesting pharmaceutical compounds classified as minor tranquilizers used in the treatment of psychoneuroses to reduce pathological anxiety, agitation and tension [1]. Nitrazepam is widely used as hypnotic. Many analytical methods have been reported for their determination in pharmaceutical formulations or in biological fluids including spectrophotometry [2], polarography [3] and HPLC [4]. Some of these methods lack of adequate detectability (require large amounts of sample), are time consuming or costly. A systematic review of published methods for the determination of benzodiazepines is included in two excellent monographs written by Schütz [5,6]. The native fluorescence of 1,4-benzodiazepines is very low [7] but their fluorescence emission can be enhanced after acidic hydrolysis [8,9] or by photodegradation [10]. The type and concentration of the acid used for hydrolysis and the presence of an alcohol (methanol or ethanol) affect the fluorescence in-

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0731-7085/99/$ - see front matter
PII: S0731-7085(99)00064-3

tensity [11]. Oxazepam shows a very intensive fluorescence in alcoholic solutions of phosphoric acid after heating at 60–100°C [12,13]. In the current European Pharmacopoeia 1,4-benzodiazepines in bulk materials are determined by non-aqueous titration [14].

Flow injection (FI) technique has gained a widespread recognition in the automation of methods used in the area of pharmaceutical analysis. It is a simple, rapid and inexpensive technique characterised by high measurement throughput, high precision, low reagent consumption and tremendous flexibility in manifold design and detectors [15–17]. Therefore, its application to routine pharmaceutical analysis has been proven to be very useful and of great potential. Recently, several FI methods have been described in the literature for the determination of 1,4-benzodiazepines. Nitrazepam was determined in tablets using voltammetric detection [18], while oxazepam was detected by fluorimetry after heating in a mixture of acetic acid and methanol to produce an intensive fluorescence product [19] or by spectrophotometry [20]. An FI method for the determination of diazepam by irradiation with Hg-lamp in alkaline medium has also been described [10]. None of these methods are fully automated FI procedures.

The aim of this work was to develop a novel, simple, rapid and fully automated method for the assay of 1,4-benzodiazepines (oxazepam, diazepam and nitrazepam) in pharmaceutical formulations using FI technique. The method is based on the formation of fluorescent products after acid hydrolysis in alcoholic medium at room temperature.

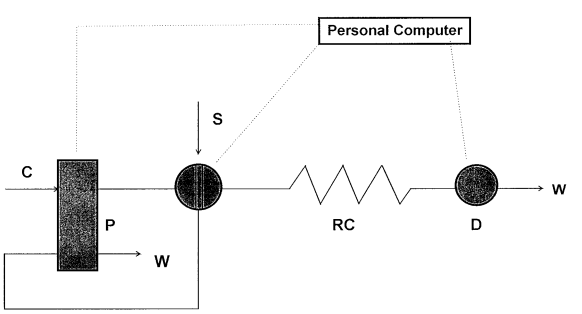


Fig. 1. Schematic diagram of the FI manifold used for the determination of 1,4-benzodiazepines. C, carrier (1.4 ml min^{-1}); S, sample (200 μl); P, peristaltic pump; W, waste; RC, reaction coil (0.5 m); D fluorescence detector.

2. Experimental

2.1. Apparatus

The FI manifold for the determination of 1,4-benzodiazepines is shown in Fig. 1. It consists of a peristaltic pump (Gilson-Minipuls 3, type M3, Gilson Medical Electronics, USA), a sample-loop injector (4-way) controlled by an electric actuator (Model 99T, Watrex, Prague, Czech Republic) and a built-in Rheodyne valve (Model 5020, Cotati, CA, USA); a fluorimeter (Spectra-Physics, type FS 970, USA) equipped with a flow cell (void volume of 8 μl) and a cut-off emission filter; an IBM compatible personal computer (486-SX, 8 MB RAM) to which the analogue output of the fluorimeter was fed using a home-made interface circuit based on a 10-bit analogue-to-digital converter (ADC 1005 CCJ, National Semiconductor, USA). The pump and the injection valve were controlled by the computer through a home-developed software (FIA-MOD 2.1). All connections and mixing coil were made from 0.5 mm i.d. PTFE tubing.

The UV-spectrophotometric determination of the drugs in the commercial formulations using the official methods was performed using an UV-VIS spectrophotometer (Pye Unicam, SP 8-200, Cambridge, UK) equipped with a 10-mm quartz cell.

2.2. Computer program

All the operations of the FI system were under computer control. The computer programme FIA-MOD 2.1 was written in Turbo-Pascal 6 and enables the control of the multichannel peristaltic pump (motor on/off, speed and direction of the flow), injection valve status (loading and sampling position with the associated timing) and signal processing from the detector (any detector with an output voltage signal in the range 0–2.5 V can be used). All experimental parameters are introduced

prior to the starting of measurements by the operator (overall period and sampling time intervals; normal and washing pump speed and the associated time intervals; time intervals for baseline measurement and integration; number of cycles; selection of the mode for baseline correction). All the current parameters along with the current position of the valve, the direction and speed of the pump are displayed during measurements on the screen.

The FI-gram obtained is displayed on the screen in real-time and it is automatically saved on the hard disc. The calculation of the peak-height or peak-area is performed automatically and the results are recorded on the printer. The algorithm used for the calculation of the peak height is based on a quadratic fit of the upper most 11 signal-time points to minimise the effect of noise spikes. Detailed description of a previous version of this program has been presented earlier elsewhere [21].

Data stored on the hard disc were then transferred to Excel and processed by packet SlideWrite 4.1. (Advanced Graphic Software, USA).

2.3. Reagents

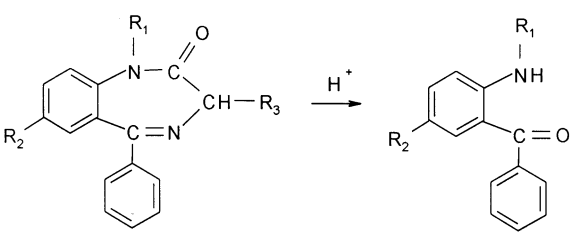
Stock standard solution of 2.5 mg ml^{-1} of oxazepam or 0.5 mg ml^{-1} of diazepam in ethanol denatured with 2% (v/v) methanol and 0.5 mg ml^{-1} of nitrazepam in methanol were prepared from the corresponding pure substances (Sigma-Aldrich, Prague, Czech Republic). The exact purity of these substances was determined using the European Pharmacopoeia methods [14]. These solutions are stable for at least three months if they are kept in a refrigerator. Working standard solutions were prepared daily by appropriate dilution of the stock solutions with the same solvent. Carrier solutions contained 0.01 or 0.05 mol l^{-1} H_2SO_4 (Lachema, Neratovice, Czech Republic) in ethanol (oxazepam, diazepam) or methanol (nitrazepam). They were filtered through a membrane filter (Synpor, Prague, pore size 0.85 (μm) and degassed under reduced pressure prior to use.

2.4. Determination of 1,4-benzodiazepines in pharmaceuticals

For the assay of solid formulations the official pharmacopoeia sampling procedure was used [22]. Ten tablets were finely powdered and weighed. From the final fine powder an accurately weighed portion was dissolved in ethanol (oxazepam, diazepam) or methanol (nitrazepam) so that the concentration of the drug was in the range of the corresponding calibration graphs. By using an ultrasonic bath the powder was completely disintegrated, the solution was filtered through a Millipore filter and directly injected (200 μl) to the FI system.

3. Results and discussion

The native fluorescence of 1,4-benzodiazepines is very low. A great enhancement of fluorescence intensity is achieved in acid media, in which the following hydrolysis process occurs [5]:



Drug	R_1	R_2	R_3
Oxazepam	H	Cl	OH
Diazepam	CH_3	Cl	H
Nitrazepam	H	NO_2	H

Several acids (sulphuric, hydrochloric and acetic) were tested for the acidic hydrolysis but only H_2SO_4 was found to enhance considerably the fluorescence emission. The use of an alcohol (methanol or ethanol) as the reaction medium is necessary due to the low aqueous solubility of

Table 1
Excitation and emission maxima used for the fluorimetric determination of 1,4-benzodiazepines

1,4-Benzodiazepine	Hydrolysing reagent (carrier)	λ_{ex} (nm)	λ_{em} (nm) (cut-off filter)
Oxazepam	0.01 mol l^{-1} H_2SO_4 in ethanol	237	418
Diazepam	0.05 mol l^{-1} H_2SO_4 in ethanol	241	370
Nitrazepam	0.05 mol l^{-1} H_2SO_4 in methanol	283	389

Table 2
Experimental variables examined in the optimization of the FI method[a]

Variable	Unit	Range studied	Optimum
Flow rate	ml min^{-1}	0.5–2.0	1.5
Mixing coil length	m	0.2–2.0	0.5
Concentration of H_2SO_4	mol l^{-1}	0.01–0.1	0.01 (diazepam and nitrazepam) 0.01 (oxazepam)
Concentration of ethanolin (in water)	% v/v	10–98	98 (oxazepam and diazepam)
Concentration of methanolin (in water)	% v/v	10–100	100 (nitrazepam)

[a] Drug concentration: oxazepam: 0.075 mg ml^{-1}; diazepam and nitrazepam: 0.050 mg ml^{-1}.

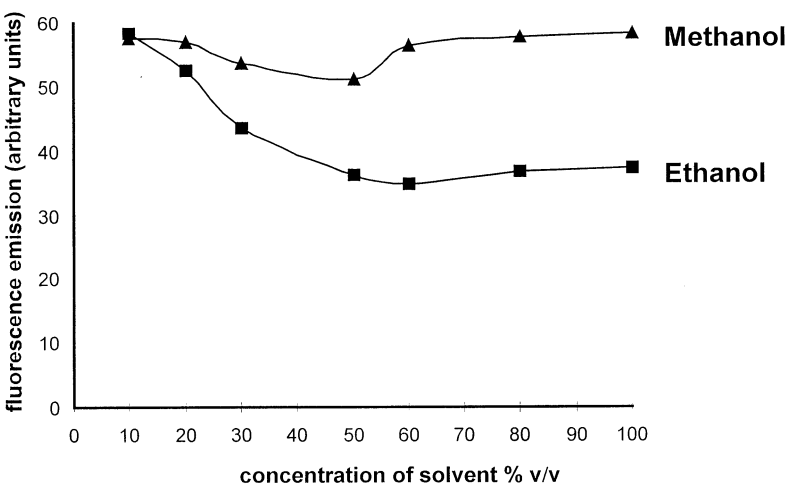


Fig. 2. Dependence of fluorescence emission of nitrazepam (0.05 mg ml^{-1}) on solvents used. $\lambda_{ex} = 283$ nm, $\lambda_{em} > 389$ nm.

drugs and the drastic enhancement of fluorescence intensity by the alcohol. The range and optimum for chemical variables is shown in Table 2.

The fluorescence excitation and emission maxima of the acidic hydrolysis product of the three drugs were measured using a fluorimeter equipped with cut-off emission filters to select the appropriate band. The results are summarised in Table 1.

3.1. Optimisation of the FI variables

In order to optimise the under development flow-injection method the univariate experimental design was used and the effect of the various experimental variables was studied with respect to sensitivity, precision and sampling rate. The range of variables studied and the optimum values cho-

sen are shown in Table 2. A fixed sample loop of 200 μl was used in the optimisation procedure and the routine measurements. Nitrazepam was found also to develop a high fluorescence emission after acid hydrolysis in a 10% (v/v) aqueous solution of ethanol (Fig. 2). However because of its limited solubility in this medium, 100% methanol was finally chosen as the solvent.

3.2. Analytical applications

Using the optimum experimental parameters of Tables 1 and 2, calibration curves were constructed for the three drugs. The linear regression equations and precision data are shown in Table 3. The linearity and precision are excellent and the detection limits (3 SD) are: oxazepam (0.01 mg ml^{-1}), nitrazepam (0.005 mg ml^{-1}) and diazepam (0.005 mg ml^{-1}). The measurement throughput is about 60 h^{-1}.

3.3. Interferences

The effect of several substances that are used as common excipients in pharmaceutical preparations on the FI determination was studied. Synthetic mixed solutions of oxazepam, diazepam or nitrazepam at the concentration level of 0.025 mg ml^{-1} and varying concentrations of the excipients were prepared and analysed. The maximum tolerance ratio (yielding an error less than (5% in the analytical signal) for glucose, sucrose, lactose, galactose and saccharin was found to be about 100 in all cases.

3.4. Assay of drugs in pharmaceutical preparations

The proposed FI method was applied for the determination of oxazepam, diazepam and nitrazepam in commercially available pharmaceuti-

Table 3
Linear regression calibration parameters and reproducibility[a] for the FI determination of oxazepam, diazepam and nitrazepam

Drug	Concentration range (mg ml^{-1})	Equation ($F_{(nA)} = a + b.c$)	r	RSD[a] (%)
Oxazepam	0.025–0.150	$F = 0.94 \pm 694.c$	0.99996	0.75
Diazepam	0.010–0.125	$F = -0.77 \pm 351.c$	0.9993	0.68
Nitrazepam	0.010–0.150	$F = 1.52 \pm 709.c$	0.9993	0.70

[a] Ten runs of 0.075 mg ml^{-1}.

Table 4
Determination of 1,4-benzodiazepines in pharmaceutical preparations

Sample (Manufacturer)	Claimed (mg)	Found (mg)[a]		t-test[c] (t_{exp})
		FIA method	Reference[b]	
Oxazepam tablets (Léčiva, Prague, Czech Republic)	10	9.81 ± 0.4	9.90 ± 0.8	0.221
Diazepam tablets (Slovakofarma, Hlohovec, Slovak Republic)	5	4.92 ± 0.4	4.95 ± 0.5	0.090
Diazepam tablets	10	9.83 ± 0.3	9.91 ± 0.8	0.200
Nitrazepam forte tablets (Slovakofarma, Hlohovec, Slovak Republic)	10	9.80 ± 0.4	9.89 ± 0.8	0.196

[a] Mean of determinations: $n_{FIA} = 5$, $n_{REF} = 3 \pm$ SD.
[b] UV spectrophotometric method.
[c] t_{teor} (95%): 2447.

cal formulations. The tablet excipients had no any effect on the determinations. The data shown in Table 4 reveals that the results of the FI method are in good agreement with those obtained by the official UV spectrophotometric (reference) method [22] (*t*-test). The disadvantage of the official direct UV- spectrophotometric method is the possible interference from excipients. FI procedure is not only more selective, but also more rapid, fully automated with a high measurement throughput, low consumption of reagents and high precision. The use of a closed FI system when using organic solvents is also an advantage.

4. Conclusion

The FI technique is suitable to automate on line wet-chemical procedures, like the acidic hydrolysis of 1,4-benzodiazepines, to produce highly fluorescent products. The very precise timing control of the FI technique ensures high precision of the analytical results. Automated FI methods are very useful for the routine quality control of pharmaceutical formulations.

Acknowledgements

The authors acknowledge: the support from the Greek Ministry of Industry, Energy and Technology and the Czech Ministry of Education for the co-operation; the Grant Agency IGA of the Ministry of Health Czech Republic, grant No. 4841-3.

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