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Direct derivative spectrophotometric determination of nitrazepam and clonazepam in biological fluids

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Abstract: The use of fifth order derivative spectra allows the direct determination of nitrazepam in urine at 388 nm with a limit of detection of 1 μg ml^{-1}. The determination of nitrazepam in blood plasma can be carried out directly by measurement at 402 nm in the fourth order derivative spectra with a limit of detection of 1.5 μg ml^{-1}. Clonazepam can be determined directly in urine samples at 384 nm by using sixth order derivative spectra with a limit of detection of 1 μg ml^{-1} and in blood plasma by using fourth order derivative spectra at 384 nm with a limit of detection of 0.5 ppm.

Keywords: *Nitrazepam; clonazepam; derivative spectrophotometry; urine analysis; blood plasma analysis.*

Introduction

Benzodiazepines are one of the most widely used group of drugs at present [1]. The therapeutic interest in these compounds justifies research to establish analytical methods for their determination in pharmaceutical products and for the investigation of physiological absorption–elimination processes [2]. In the analysis of pharmaceutical products the matrix is relatively simple and usually there are no sensitivity problems. Also, in cases of benzodiazepine abuse, relatively high concentrations of benzodiazepine are found in biological samples. However, after administration of therapeutic doses concentrations of the drugs in biological samples are low and a sensitive method must be used. Usually, extractions or other separation stages are carried out to avoid matrix interferences.

Methods proposed for the determination of benzodiazepines include liquid chromatography [3, 4], polarography [5, 6], atomic absorption spectrophotometry [7] and molecular spectroscopic techniques such as fluorimetry [8, 9], infrared spectroscopy [10], phosphorimetry [11] and UV spectrophotometry.

UV spectrophotometry is not a selective method because many benzodiazepines exhibit very similar spectra. The simultaneous determination of benzodiazepines in mixtures may be carried out after a derivatization reaction to generate the corresponding benzophenones followed by their spectrophotometric determination in micellar media [12]. Derivative spectroscopy has been used to determine benzodiazepines in mixtures with benzophenones after a rapid acid hydrolysis in a microwave oven [13].

Theoretical and practical aspects of derivative spectroscopy have been discussed by several authors [14–18]. Derivatization methods improve the selectivity of UV–visible spectroscopic measurements, by means of spectral deconvolution [19, 20], and they also reduce matrix effects [21, 22]. Several papers have been published on the use of derivative spectrophotometry for this purpose but, in general, when derivative spectrophotometry has been applied to the analysis of substances in biological matrices, it is employed after a separation or purification step [23, 24]. Derivative spectroscopy after preliminary separation by extraction and chromatography has been used to determine benzodiazepines in biological fluids [25].

In this paper, a method is proposed for the direct determination of benzodiazepines in biological fluids by derivative spectrophotometry. This method eliminates the background absorption of the matrix and avoids the need for a preliminary clean-up stage.

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Experimental

Apparatus

A Hewlett Packard 8452A diode array spectrophotometer equipped with a Vectra ES/12 computer was used with a 1 cm quartz cell. The system can mathematically generate up to the ninth derivative spectrum.

Reagents

Aqueous standard solutions containing 2, 4, 8, 12, 16 and 20 μg ml^{-1} of nitrazepam or clonazepam in 0.1 M sodium hydroxide were prepared from 100 μg ml^{-1} stock solutions in 0.1 M sodium hydroxide.

Spiked samples were prepared by adding known amounts of nitrazepam or clonazepam to diluted urine or blood plasma samples, in 0.1 M sodium hydroxide.

Procedure

Samples and standard spectra were recorded over a wavelength range of 226–476 nm with an integration time of 5 s. The absorption spectra were then mathematically differentiated to generate the first to the ninth derivative spectra. Wavelengths at which the derivative spectra of samples and standards with the same concentration coincide were selected for each derivative order. Concentrations of benzodiazepines in the spiked samples were determined at the selected wavelengths using calibration data obtained from aqueous standard solutions containing nitrazepam or clonazepam. Appropriate analytical parameters were determined for the analysis of both urine and blood plasma samples.

Results and Discussion

Determination of nitrazepam

Figure 1 shows the absorption spectra of spiked samples of urine and blood plasma and of an aqueous standard solution each containing 8 μg ml^{-1} of nitrazepam. The strong background absorption of the biological matrices masks the benzodiazepine spectrum and renders a direct determination impossible. The first to the ninth derivative spectra were recorded for a series of standard solutions and also for spiked samples containing known concentrations of nitrazepam. Peaks which had the same intensity and shape in the spectra of the standard and sample solutions with the same concentration were selected for each

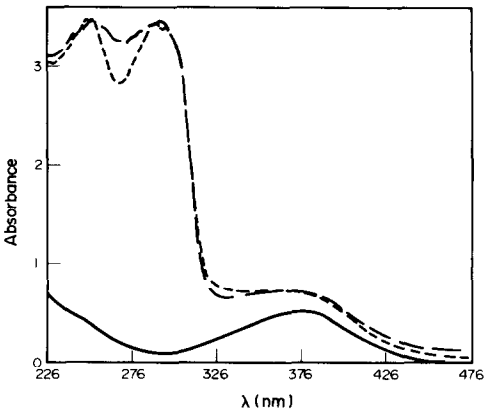

Figure 1
Absorption spectra of an aqueous standard solution of nitrazepam (—), a spiked urine sample (--) and a spiked blood plasma sample (– –). The concentration of nitrazepam in each solution was 8 μg ml^{-1}.

derivative order. In each case, the degree of interference in the analysis of nitrazepam arising from the biological matrices was determined to establish the most appropriate conditions for the direct determination of the drug.

Determination of nitrazepam in urine. The best results were obtained by using the fifth order derivative spectra. Figure 2a shows the spectra of a spiked urine sample and an aqueous standard solution of nitrazepam both containing 8 μg ml^{-1} of the benzodiazepine. Both derivative spectra were coincident in the wavelength range 360–420 nm. The peak at 388 nm gave percentage recovery values in the range 99–106% and relative standard deviations in the range 3–9% (Table 1). A good regression coefficient was obtained for the calibration line. The limit of detection, calculated by the expression LOD = 3Sb/*b* (where Sb is the standard deviation of 10 derivative values of the blank solution and *b* is the slope of the calibration line), was 0.4 μg ml^{-1} of nitrazepam in the final sample solution. Thus, for a sample of urine diluted 2:5 (2 + 3), the limit of detection was 1 μg ml^{-1}.

As some of the spectrophotometers currently used in laboratories measure only to the fourth derivative order, data for a peak in the fourth derivative spectrum, at 384 nm, are also given in Table 1. Figure 2b shows the fourth derivative spectra of an aqueous standard solution of nitrazepam and of a spiked urine sample both containing 8 μg ml^{-1}. A good regression coefficient was obtained for the

Table 1
Derivative spectrophotometric analysis of nitrazepam in urine

			Sample dilution levels								
			1:10		1:5		2:5				
Derivative order	Wavelength (nm)	Concn added ($\mu g\ ml^{-1}$)	Concn found ($\mu g\ ml^{-1}$)	Recovery (%)	Concn found ($\mu g\ ml^{-1}$)	Recovery (%)	Concn found ($\mu g\ ml^{-1}$)	Recovery (%)	RSD (%)	Calibration line	LOD‡ ($\mu g\ ml^{-1}$)
5	388	8	8.1	101	8.2	102	7.9	99	3–9	*	0.4
		16	16.5	103	16.4	103	17.0	106			
4	384	8	8.5	106	9.2	114	9.8	122	2–5	†	0.3
		16	16.8	105	17.2	107	18.2	113			

*$d = 8.69 \times 10^{-8}C + 2.05 \times 10^{-8}$, $r = -0.9997$.
†$d = 4.29 \times 10^{-7}C - 6.51 \times 10^{-8}$, $r = 0.9998$: (2–20 $\mu g\ ml^{-1}$).
d = derivative of the absorbance. C = concentration in $\mu g\ ml^{-1}$, r = linear regression coefficient.
In this and the other tables:
‡Limit of detection (LOD) for $k = 3$ ($P = 95\%$). N = number of independent samples analysed (10 measurements on each).

Table 2
Derivative spectrophotometric analysis of nitrazepam in blood plasma

			Sample dilution levels								
			1:10		1:5		2:5				
Derivative order	Wavelength (nm)	Concn added ($\mu g\ ml^{-1}$)	Concn found ($\mu g\ ml^{-1}$)	Recovery (%)	Concn found ($\mu g\ ml^{-1}$)	Recovery (%)	Concn found ($\mu g\ ml^{-1}$)	Recovery (%)	RSD (%)	Calibration line	LOD ($\mu g\ ml^{-1}$)
4	402	8	8.1	101	7.7	97	6.1	77	3–9	*	0.3
		16	16.4	102	16.0	100	19.6	123			

*$d = 2.71 \times 10^{-7}C - 1.76 \times 10^{-9}$, $r = 0.9993$ (2–20 $\mu g\ ml^{-1}$).
d = derivative of absorbance, C = concentration in $\mu g\ ml^{-1}$, r = linear regression coefficient.

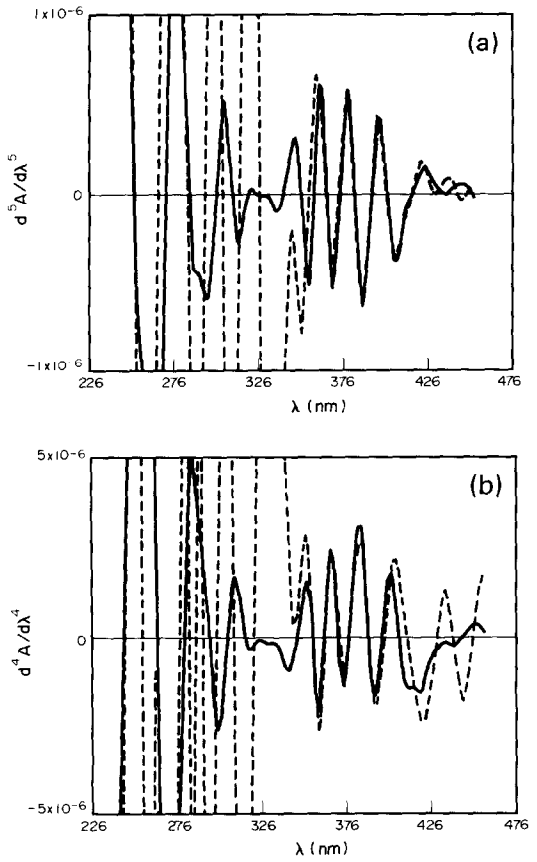

Figure 2
(a) Fifth derivative spectra of an aqueous standard solution of nitrazepam (—) and a spiked urine sample (--). The concentration of nitrazepam in both solutions was 8 μg ml^{-1}. (b) Fourth derivative spectra of an aqueous standard solution of nitrazepam (—) and a spiked urine sample (--). The concentration of nitrazepam in both solutions was 8 μg ml^{-1}.

calibration line in the fourth order derivative and the sensitivity was higher than that obtained by using the fifth derivative spectrum. However, the percentage recovery values showed that there was considerable interference from the sample matrix except when a dilution factor of 1 + 9 was used. Thus the detection limit of 0.3 μg ml^{-1} in the diluted 1:10 solution corresponds to a minimum detectable concentration of 3 μg ml^{-1} in the urine sample.

Determination of nitrazepam in blood plasma. Figure 3 shows the fourth derivative spectra of a spiked blood plasma sample and of an aqueous solution both containing 8 μg ml^{-1} of nitrazepam. The data for the peak at 402 nm, which provides the best results, are shown in Table 2. A good regression coefficient was obtained and the limit of detection,

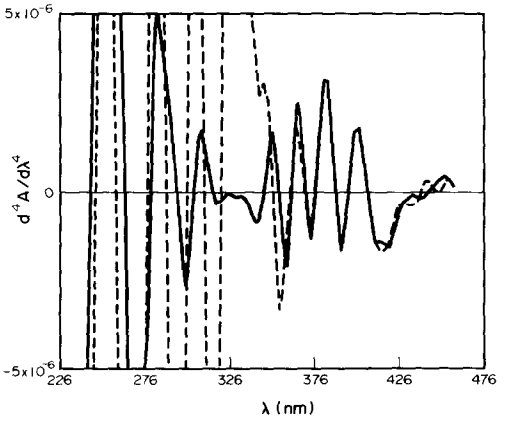

Figure 3
Fourth derivative spectra of an aqueous standard solution of nitrazepam (—) and a spiked blood plasma sample (--). The concentration of nitrazepam in both solutions was 8 μg ml^{-1}.

0.3 μg ml^{-1} in the diluted solution, corresponds to 1.5 μg ml^{-1} in the undiluted blood sample if a 1:5 dilution is used in the determination.

Determination of clonazepam

Figure 4 shows the absorption spectra of an aqueous standard solution of clonazepam and of spiked samples of urine and blood plasma. Owing to the extensive matrix interference in these spectra, the direct determination of clonazepam is not possible. The optimum conditions for the determination of clonazepam were determined by examining the derivative spectra.

Determination of clonazepam in urine. Figure 5a shows that the sixth derivative

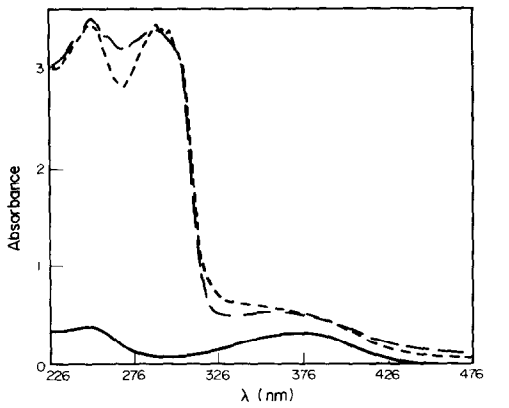

Figure 4
Absorption spectra of an aqueous standard solution of clonazepam (—), a spiked urine sample (--) and a blood plasma sample (– –), each containing 8 μg ml^{-1}.

Table 3
Derivative spectrophotometric analysis of clonazepam in urine

			Sample dilution levels								
			1:10		1:5		2:5				
Derivative order	Wavelength (nm)	Concn added ($\mu g\ ml^{-1}$)	Concn found ($\mu g\ ml^{-1}$)	Recovery (%)	Concn found ($\mu g\ ml^{-1}$)	Recovery (%)	Concn found ($\mu g\ ml^{-1}$)	Recovery (%)	RSD (%)	Calibration line	LOD ($\mu g\ ml^{-1}$)
6	384	8	7.7	97	7.9	99	8.1	102	2–12	*	0.4
		16	16.9	106	16.7	105	15.5	97			
3	408	8	7.8	97	7.4	93	7.1	89	0.7–2	†	0.1
		16	16.2	101	15.8	99	15.2	95			

*$d = 2.20 \times 10^{-8}C - 8.35 \times 10^{-9}$, $r = 0.9996$.
†$d = 2.95 \times 10^{-6}C - 1.10 \times 10^{-6}$, $r = 0.9999$ (2–20 $\mu g\ ml^{-1}$).
d = derivative of the absorbance. C = concentration in $\mu g\ ml^{-1}$, r = linear regression coefficient.

Table 4
Derivative spectrophotometric analysis of clonazepam in blood plasma

			Sample dilution levels										
			1:10		1:5		2:5		3:5				
Derivative order	Wavelength (nm)	Concn added ($\mu g\ ml^{-1}$)	Concn found ($\mu g\ ml^{-1}$)	Recovery (%)	Concn found ($\mu g\ ml^{-1}$)	Recovery (%)	Concn found ($\mu g\ ml^{-1}$)	Recovery (%)	Concn found ($\mu g\ ml^{-1}$)	Recovery (%)	RSD (%)	Calibration line	LOD ($\mu g\ ml^{-1}$)
4	384	8	7.7	96	7.2	91	6.5	81	7.1	89	2–12	*	0.1
		16	15.9	99	15.4	96	13.5	84	15.1	94			

*$d = 4.21 \times 10^{-7}C - 1.13 \times 10^{-7}$, $r = 0.9998$ (2–20 $\mu g\ ml^{-1}$).
d = derivative of absorbance, C = concentration in $\mu g\ ml^{-1}$, r = linear regression coefficient.

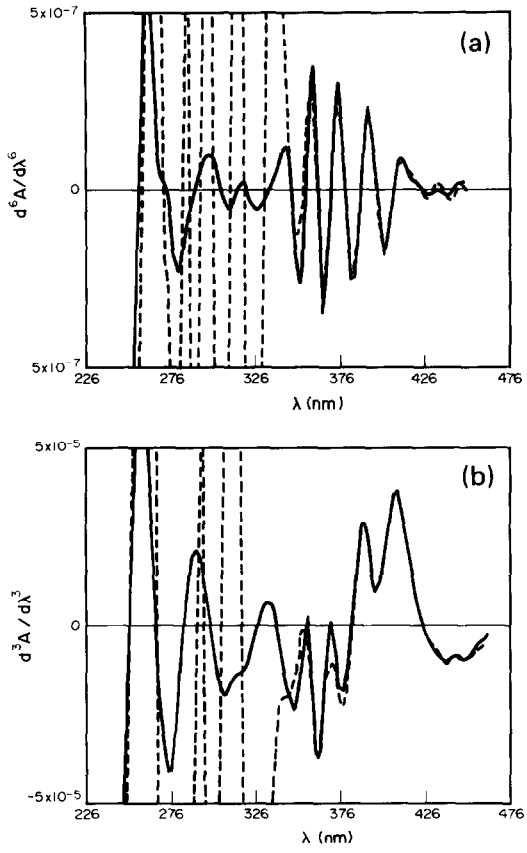

Figure 5
(a) Sixth derivative spectra of a standard solution of clonazepam (—) and a spiked urine sample (--) both containing 8 μg ml^{-1}. (b) Third order derivative spectra of a standard solution of clonazepam (—) and a spiked urine sample (--) both containing 8 μg ml^{-1}.

spectra of an aqueous standard solution of clonazepam and of a spiked sample of urine, both containing 8 μg ml^{-1}, are concordant over the wavelength range 350–420 nm. Table 3 shows that percentage recoveries of 97–106% were obtained with a RSD of 2–12% when a wavelength of 384 nm was used. The limit of detection was 0.4 μg ml^{-1} in the diluted (1:5) solution corresponding to 1 μg ml^{-1} in the undiluted sample.

The possibility of using a lower order derivative was investigated. Table 3 shows that data obtained at a wavelength of 408 nm in the third order derivative spectra (Fig. 5b) provides a good regression coefficient of the calibration line with a limit of detection of 0.1 μg ml^{-1} in the diluted solution corresponding to 0.25 μg ml^{-1} in the undiluted urine sample if a 2:5 dilution is used for the determination. However errors were higher than those obtained by using the sixth derivative spectra as shown by the percentage recovery values.

Determination of clonazepam in blood plasma. The fourth derivative spectra of an aqueous solution of clonazepam and of a spiked sample of blood plasma both containing 8 μg ml^{-1} are shown in Fig. 6. At a wavelength of 384 nm, percentage errors of less than 10% were found when a sample dilution of 2:5 was carried out, with RSD values of 2–12% (Table 4).

A limit of detection of 0.1 μg ml^{-1} in the diluted (1:5) solution corresponding to 0.5 μg ml^{-1} in the undiluted sample was obtained.

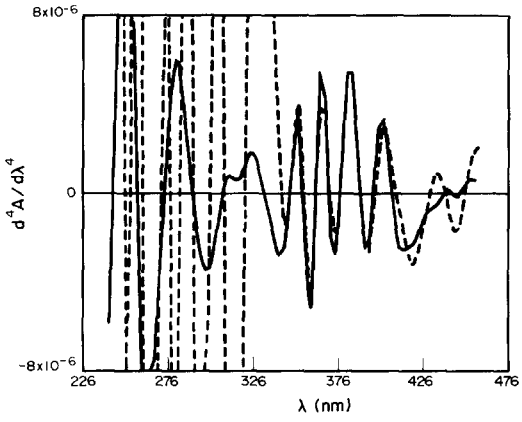

Figure 6
Fourth order derivative spectra of a standard solution of clonazepam (—) and a spiked blood plasma sample (--) both containing 8 μg ml^{-1}.

Conclusion

The use of UV derivative spectra allows the direct determination of nitrazepam and clonazepam in biological fluids at the μg ml^{-1} level without any extraction or separation step. The method avoids the matrix effects that occur at wavelength higher than 300 nm. In this region the endogenous components of urine and blood plasma interfere significantly in the fundamental absorption spectra but not in the higher order derivative spectra. Appropriate wavelengths and derivative orders must be used to obtain good accuracy and reproducibility. The results obtained in the present study demonstrate that the proposed method is more sensitive and accurate than those previously published for the derivative spectrophotometric determination of benzodiazepine compounds. However, excessive pigmentation of urine samples may result in errors.

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