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Determination of new 2,3-benzodiazepines in rat plasma using high-performance liquid chromatography with ultraviolet detection

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Abstract

A method for the analysis of [1-(4-aminophenyl)-3,5-dihydro-7,8-dimethoxy-4H-2,3-benzodiazepin-4-one] (CFM-2) and its analogues CFM-3, CFM-4 and CFM-5 in rat plasma was developed. The 2,3-benzodiazepines (2,3-BZs) were extracted by liquid–liquid extraction and analyzed using high-performance liquid chromatography (HPLC) with ultraviolet detection (UV) at 240 nm. The method exhibited a large linear range from 0.05 to 2 μg/ml with an intra-assay accuracy for all studied compounds ranging from 92 to 105.5%; whereas the intra-assay precision ranged from 0.59 to 8.16% in rat plasma. The inter-assay accuracy of CFM-2, CFM-4 and their 3-methyl derivatives, CFM-3 and CFM-5 ranged from 92.2 to 107% and the inter-assay precision ranged from 2.17 to 11.9% in rat plasma. The lower limit of detection was 5.5 ng/ml for CFM-2, 6.5 ng/ml for CFM-3, 7 ng/ml for CFM-4 and 8.5 ng/ml for CFM-5 in rat plasma. The pharmacokinetic study demonstrated that 2,3-BZs achieved a peak plasma concentration between 45 and 75 min after drug administration. Moreover, we observed that plasma chromatograms of rats treated with CFM-3, CFM-4 and CFM-5, respectively, showed a peak consistent with CFM-2. Our study suggests that CFM-4, CFM-5 and CFM-3 are prodrugs of CFM-2, in which they are biotransformed in vivo via different metabolic pathways. In particular, CFM-2 has been proven to possess anticonvulsant activity in various models of seizures, acting as α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) receptor antagonist.

Keywords: Benzodiazepines

1. Introduction

The pathogenesis of different neurological disorders has been linked to excessive activation of excitatory amino acid (EAA) receptors. The development of therapeutically useful *N*-methyl-D-aspartic acid (NMDA) receptor channel blockers and a new class of selective non-NMDA receptor antagonists i.e., α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA)/kainate, may be useful in the treatment of various disorders such as epilepsy, ischaemia and Parkinson's disease [1]. The 2,3-benzodiazepines (2,3-BZs) represent a new class of selective AMPA/kainate receptor antagonist.

GYKI 52466 is a 2,3-BZ derivative which differs

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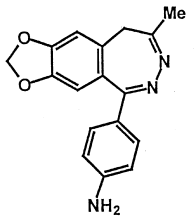
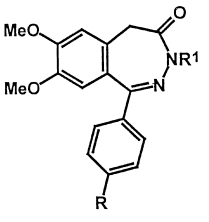
pharmacologically from classical 1,4- and 1,5-benzodiazepines in that it possesses muscle relaxant and anticonvulsant properties acting as a highly selective non-competitive antagonist at the AMPA/kainate receptor site and shows no affinity for the benzodiazepine receptors (BZRs) [2,3]. Recently, new series of 2,3-BZs chemically similar to GYKI 52466 (Fig. 1) were synthesized in our laboratories and proved to possess anticonvulsant activity in various experimental models of seizures [4,5]. It has been demonstrated that these 2,3-BZ derivatives are non-competitive antagonists at the AMPA/kainate receptor and do not affect NMDA and γ-aminobutyric acid (GABA) receptor-mediated responses [5]. Particularly, [1-(4-aminophenyl)-3,5-dihydro-7,8-dimethoxy-4H-2,3-benzodiazepin-4-one] (CFM-2) has a marked anticonvulsant activity comparable to that of GYKI 52466 and its effect may last longer than GYKI 52466. Besides the CFM-2 and its 3-methyl derivative CFM-3, other compounds, i.e., CFM-4, [3,5-dihydro-7,8-dimethoxy-1-(4′-nitrophenyl)-4H-2,3-benzodiazepin-4-one] and its 3-methyl derivative CFM-5, have been synthesized, and are object of our study.

In the present study we have developed an assay suitable for simultaneous determination of 2,3-BZs in rat plasma by using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection.

Our goal was to develop a sensitive method to detect drug concentrations of the order of nanograms in only 500 μl of plasma.



GYKI 52466



CFM-2	R= NH_2	R^1 = H
CFM-3	R= NH_2	R^1 = Me
CFM-4	R= NO_2	R^1 = H
CFM-5	R= NO_2	R^1 = Me
I.S.	R= H	R^1 = Me

Fig. 1. Structures of GYKI 52466, 2,3-BZs and I.S.

2. Experimental

2.1. Chemicals and standards

Ethyl acetate, acetonitrile, methanol and water HPLC-grade (Carlo Erba, Milan, Italy) were used and deionized water was used for HPLC buffer. All other reagents were of analytical-reagent grade (Carlo Erba). The internal standard (I.S.), was 3,5-dihydro-7,8-dimethoxy-3-methyl-1-phenyl-4H-2,3-benzodiazepin-4-one) (Fig. 1) and was prepared at a concentration of 10 μg/ml. Compounds CFM-2 (now available from Tocris Cookson, Ballwin, MO, USA), CFM-3 [1-(4′-aminophenyl)-3,5-dihydro-7,8-dimethoxy-3-methyl-4H-2,3-benzodiazepin-4-one], CFM-4 and CFM-5 [3,5-dihydro-7,8-dimethoxy-1-(4′-nitrophenyl)-3-methyl-4H-2,3-benzodiazepin-4-one] (Fig. 1) were synthesized in our laboratories as previously described [5]. Stock solutions (1 mg/ml) of all 2,3-benzodiazepines were prepared in acetonitrile. Working solutions were made by dilution with methanol and used to prepare aqueous standards and spiked plasma samples on a standard curve.

2.2. Chromatography

The HPLC system consisted of a Beckman Analytical (Cassina De Pecchi, Milan, Italy) System Gold 125 solvent module with a 20-μl loop injection valve, a variable-wavelength UV 166 Detector set at 240 nm and a Epson Endeavoir 4DX2/50 L integrator. A Partisil 10 ODS Whatman (Carlo Erba) (250×4.6 mm I.D.) reversed-phase column was used with an ODS guard (45×4.6 mm). The mobile phase consisted of acetonitrile–0.01 *M* acetate buffer, pH 5.25 (adjusted with pure acetic acid) (35:65, v/v) at a flow-rate of 2 ml/min. The separations were performed at room temperature [6].

2.3. Drug administration

The 2,3-BZ derivatives were dissolved in a solution containing 50% dimethyl sulfoxide and 50% sterile saline, and administered intraperitoneally (i.p.) to Sprague–Dawley rats (10 mg/kg body mass). Blood samples were withdrawn from the animals by an indwelling catheter, at 15, 30, 45, 60, 75, 90, 105, 120, 135 and 150 min after i.p administration of the

drugs. Blood cells were removed by centrifugation and the separated plasma was stored at −20°C until assay.

2.4. Extraction

A 500-μl aliquot of plasma was mixed with 100 μl of 2 *M* NaOH and 100 μl of I.S. (10 μg/ml). All standard and spiked samples were similarly treated. The sample was applied to an Extrelut 1 (E. Merck, Darmstadt, Germany) pre-packed glass column. After 10 min, ethyl acetate (10 ml) was added to the column. The eluate was collected and evaporated to dryness at 45°C under a stream of nitrogen. The residue was dissolved in 100 μl of mobile phase and a 20-μl aliquot was injected into the chromatographic system.

2.5. Calibration curve and method validation

CFM-2, CFM-3, CFM-4 and CFM-5 (0.5 μg) and I.S. (1 μg), were injected into the column and identified from their relative retention times. Calibration curves were obtained by plotting the peak area ratio of the drugs to the I.S., versus the known concentration of each reference substance added to drug-free rat plasma. The curves were constructed from six replicate measurements of six concentrations of each compound over the range 0.05 to 2 μg/ml, all performed in the same day. Plasma samples containing a known amount of all studied compounds and I.S. were prepared and stored frozen at −20°C until use. These samples were utilized as quality control specimens. The within-day and between-day precision and accuracy were calculated by six replicate analysis at each concentration (0.1–0.5–1 μg/ml) utilized and analyzed once a week for six weeks.

2.6. Extraction efficiency and stability

The determination of the extraction efficiency in rat plasma was made by adding amounts of 0.1, 0.3 and 0.5 μg/ml without internal standard. The extraction was conducted as described above and 100 μl of internal standard working solution was added to the eluate after extraction. The peak-area ratios were compared to the ratio of the standard aqueous

Table 1
Within-day precision of the HPLC assay (n=6)

Nominal concentration (μg/ml)	Measured concentration (Mean±SD) (μg/ml)	Accuracy (%)	RSD (%)
CFM-2			
0.1	0.0943±0.005	94.3	5.3
0.5	0.504±0.003	100.8	0.59
1	1.026±0.028	102.6	2.72
CFM-3			
0.1	0.092±0.002	92	2.17
0.5	0.049±0.004	98.9	8.16
1	1.00±0.029	100	2.90
CFM-4			
0.1	0.096±0.007	96.2	7.29
0.5	0.464±0.013	92.9	2.80
1	0.979±0.029	97.9	3.06
CFM-5			
0.1	0.103±0.005	103	4.4
0.5	0.53±0.036	105.5	6.8
1	0.98±0.012	97.5	1.32

RSD=Relative standard deviation.

Table 2
Between-day precision of the HPLC assay ($n=6$)

Nominal concentration (μg/ml)	Measured concentration (Mean±SD) (μg/ml)	Accuracy (%)	RSD (%)
CFM-2			
0.1	0.107±0.011	107	10.2
0.5	0.53±0.036	105.5	6.8
1	0.969±0.048	96.9	5.0
CFM-3			
0.1	0.097±0.005	96.9	5.6
0.5	0.51±0.039	101.8	7.8
1	0.99±0.022	99.01	2.22
CFM-4			
0.1	0.096±0.007	96.2	6.8
0.5	0.50±0.023	100.9	4.7
1	0.94±0.053	93.7	5.7
CFM-5			
0.1	0.098±0.006	97.6	6.4
0.5	0.49±0.058	98.8	11.9
1	0.92±0.02	92.2	2.17

RSD=Relative standard deviation.

Table 3
Determination of extraction recovery of 2,3-benzodiazepines from plasma ($n=6$)

Nominal concentration (μg/ml)	Peak-area ratio,		Calculated extraction efficiency (%)
	After extraction	Of aqueous standard	
CFM-2			
0.1	0.1211	0.1300	93.15
0.3	0.3543	0.3799	93.26
0.5	0.6018	0.6485	92.79
CFM-3			
0.1	0.1103	0.1212	91.00
0.3	0.2866	0.3085	92.90
0.5	0.4777	0.5095	93.75
CFM-4			
0.1	0.0506	0.0598	84.61
0.3	0.1377	0.1499	91.86
0.5	0.3506	0.3982	88.04
CFM-5			
0.1	0.0438	0.0589	74.36
0.3	0.1144	0.1259	90.86
0.5	0.2538	0.2775	91.45

samples without extraction. Six replicate samples were determined at each point and analysis was performed once a day for six days.

The stability of the 2,3-BZs (0.05 and 1 μg/ml) in rat plasma was studied after two, four and 10 weeks of storage at −20°C. The I.S. was added immediately before the extraction of the samples. Three replicate determinations were made in each case and at each time for each substance.

2.7. Mass spectra

Mass spectra were recorded on a Finnigan Mat 90 instrument (ThermoQuest, Rodano, Milan, Italy), in the electron impact (70 eV) mode.

3. Results and discussion

3.1. Detection and sensitivity

The retention times were 3.4 (±0.015) min for CFM-2, 4.7 (±0.010) min for CFM-3, 6.8 (±0.017) min for CFM-4, 11.5 (±0.021) min for CFM-5 and 8.6 (±0.015) min for I.S. The lower limit of detection (LOD), with a signal-to-noise ratio of 3, was 5.5 ng/ml for CFM-2, 6.5 ng/ml for CFM-3, 7.0 ng/ml for CFM-4 and 8.5 ng/ml for CFM-5. The sensitivity of the method allowed an easy quantitation of 20 ng (limit of quantitation, LOQ) of these drugs in plasma samples.

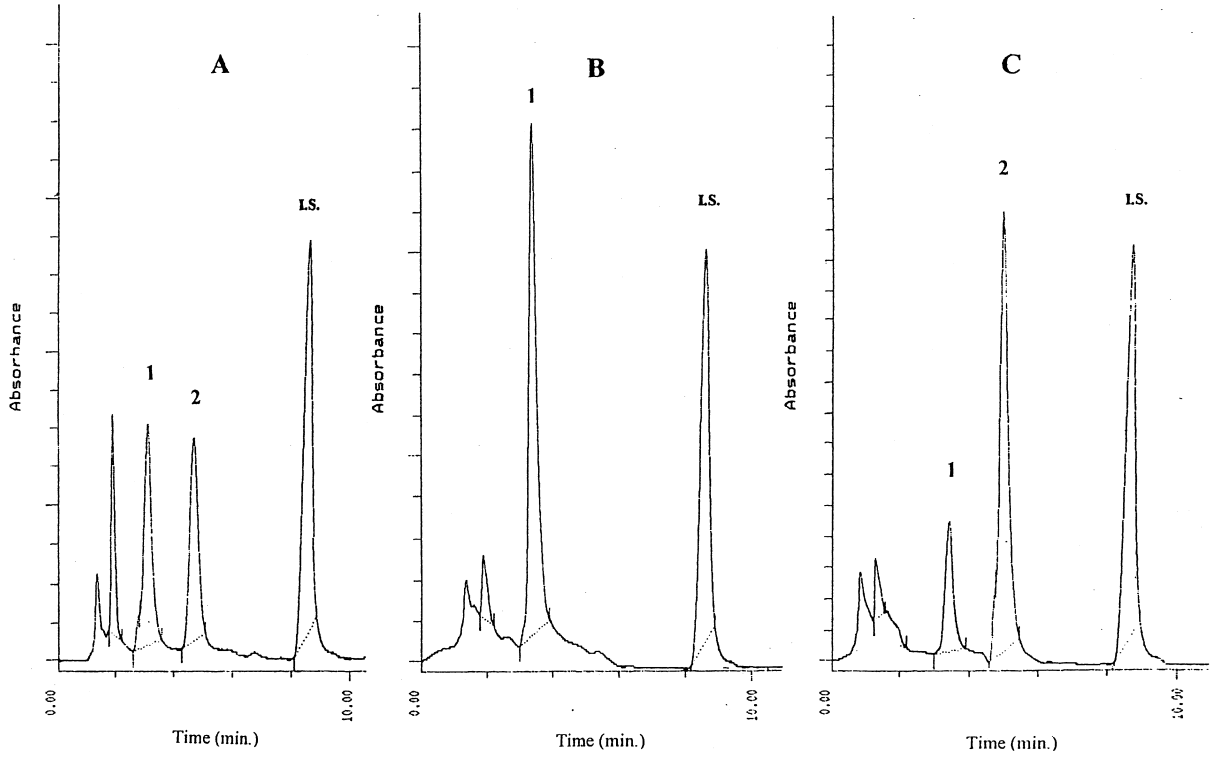


Fig. 2. Representative chromatograms of 2,3-BZs and the internal standard used in the analytical procedure. The relative retention times are: t'=3.4 for CFM-2 (1); t'=4.7 for CFM-3 (2) and t'=8.6 min for internal standard. (A) Chromatograms of rat plasma spiked with CFM-2 (0.5 μg/ml), CFM-3 (0.5 μg/ml) and I.S. (B) Chromatograms of a plasma sample obtained 45 min after administration of CFM-2 spiked with I.S. (C) Chromatograms of a plasma sample obtained 45 min after administration of CFM-3 spiked with I.S. (bis) Blank control plasma extract.

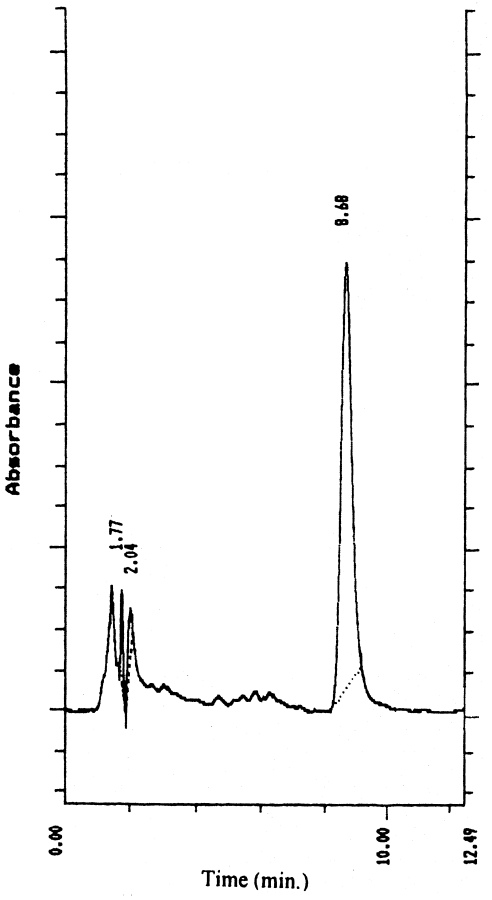


Fig. 2. (*continued*)

3.2. Linearity

A linear response was observed over the examined concentration range (0.05 to 2 μg/ml). The regression coefficient was 0.9957 for CFM-2 ($y=0.6411x+0.1178$, $n=6$); 0.9597 for CFM-3 ($y=0.9075x+0.0438$, $n=6$); 0.9696 for CFM-4 ($y=1.3363x+0.0126$, $n=6$); and 0.999 for CFM-5 ($y=2.3636x-0.0046$, $n=6$).

3.3. Precision and accuracy

Within-day and between-day precision and accuracy were performed. The results are shown in Tables 1 and 2. For all 2,3-BZs the average relative standard deviation (RSD) value analysis was 3.95% for within-day assay and 6.25% for between-day assay. Acceptable accuracy was achieved for all concentrations investigated. In the between-day assay the accuracy ranged from 92.2 to 107%, which is adequate for biological samples.

The recovery from plasma and aqueous standards of all compounds and the I.S. was evaluated to test the efficiency and reproducibility of the extraction procedure. The compounds were added to drug-free plasma to achieve concentrations of 0.1, 0.3 and 0.5 μg/ml ($n=6$ at each concentration). The recovery ranged from 74.36 to 93.75% and was independent of the concentration, as reported in Table 3, except for CFM-5.

All 2,3-BZs studied showed no signs of significant degradation in rat plasma during 10 weeks of storage at −20°C.

3.4. Application to pharmacokinetic study

The present method has been applied to the pharmacokinetic study of CFM-2, CFM-3, CFM-4 and CFM-5 after i.p. administration to Sprague–Dawley rats.

Fig. 2A shows chromatograms from drug-free rat plasma spiked with CFM-2, CFM-3 and I.S.; Fig. 2B and C show chromatograms from rat plasma samples obtained 45 min after administration of CFM-2 and CFM-3 spiked with I.S. In the second chromatogram a peak due to CFM-2 is observed (Fig. 2B). In the third chromatogram a peak due to CFM-3 and another peak corresponding to CFM-2 can be observed (Fig. 2C). This suggested that CFM-3 was

Table 4
Mass spectral data of CFM derivatives

Compound	*m/z* (%)
CFM-2	311 (M^+, 100), 282 (48), 268 (7), 254 (7), 238 (7)
CFM-3	325 (M^+, 100), 296 (23), 282 (64), 254 (8), 238 (7)
CFM-4	341 (M^+, 100), 327 (21), 312 (67), 311 (20), 296 (10), 282 (7), 266 (22)
CFM-5	355 (M^+, 100), 326 (26), 312 (79), 311 (20), 296 (10), 282 (7), 266 (22)

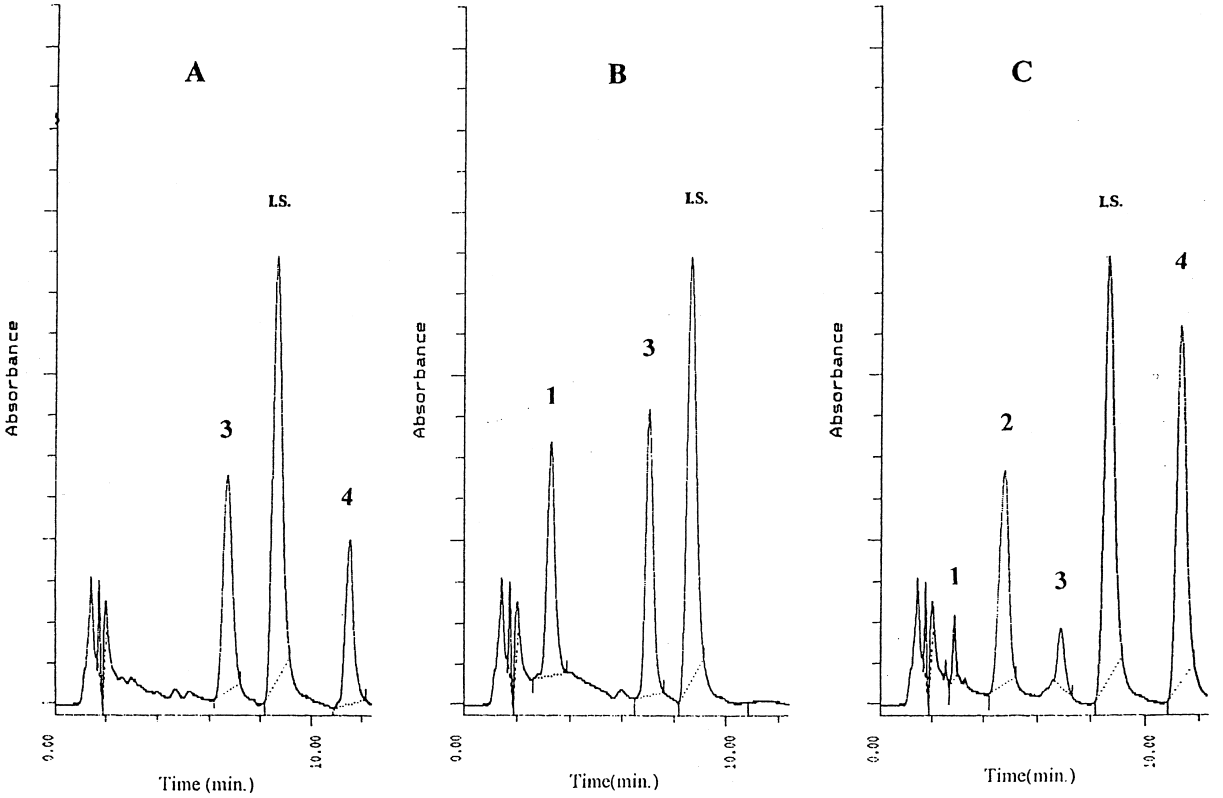


Fig. 3. Representative chromatograms of 2,3-BZs and the internal standard used in the analytical procedure. The relative retention times are: $t'=3.4$ for CFM-2 (1); $t'=4.7$ for CFM-3 (2); $t'=6.8$ for CFM-4 (3); $t'=11.5$ for CFM-5 (4) and $t'=8.6$ min for internal standard. (A) Chromatograms of rat plasma spiked with CFM-4 (0.5 μg/ml), CFM-5 (0.5 μg/ml) and I.S. (B) Chromatograms of a plasma sample obtained 75 min after administration of CFM-4 spiked with I.S. (C) Chromatograms of a plasma sample obtained 75 min after administration of CFM-5 spiked with I.S.

partially converted in vivo into CFM-2. Fig. 2 bis shows a chromatogram of a blank extract.

Fig. 3A shows chromatograms from drug-free rat plasma spiked with CFM-4, CFM-5 and I.S.; Fig. 2B and C show chromatograms from rat plasma samples obtained 75 min after administration of CFM-4 and CFM-5 spiked with I.S. In the second chromatogram a peak due to CFM-4 and another peak corresponding to CFM-2 can be observed (Fig. 3B). This suggested that CFM-4 undergoes biotransformation in vivo into CFM-2. In the third chromatogram four peaks of interest were observed, corresponding not only to CFM-5 but also to CFM-3 and their demethylated derivatives (Fig. 3C). These features suggested again that CFM-5 was partially converted in vivo into CFM-2.

The biotransformation in vivo of CFM-4 and CFM-5 was due to a reduction of the NO_2-substituent of the aryl ring at the C-1 position into the NH_2 group. Moreover, the biotransformation in vivo of CFM-3 and CFM-5 to the corresponding demethylated compounds was due to the loss of methyl group at the N-3 position, as already demonstrated for other 2,3-BZs [7], and other benzodiazepine derivatives [8], by the oxidation reaction in the liver microsomes [9].

All the above mentioned biotransformations were confirmed by mass spectra recorded after collection of the different fractions from HPLC analysis. The mass spectra showed correct molecular ions which were the base peak in all derivatives and fragmentations consistent with the proposed structures (Table 4).

Fig. 4 shows the time profiles of plasma con-

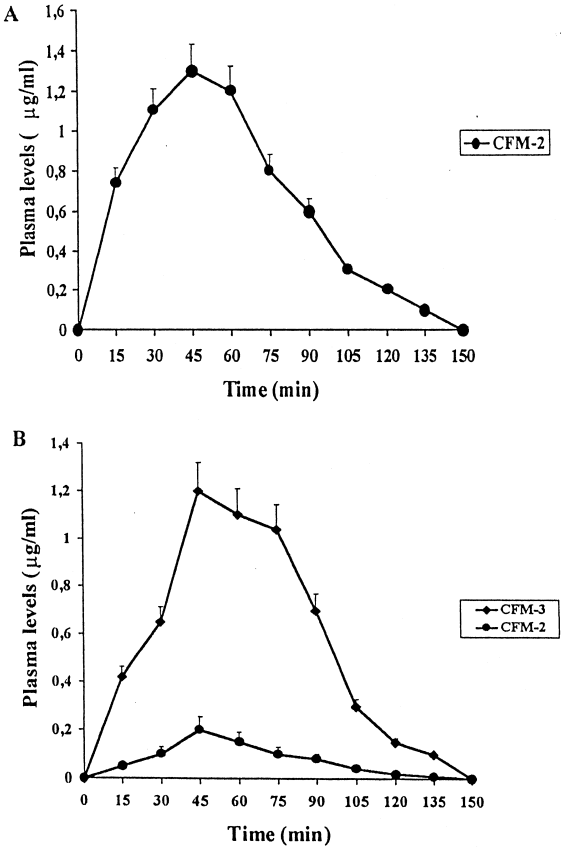


Fig. 4. Time profiles of plasma concentrations after i.p. administration in rats of CFM-2 (A) and CFM-3 (B). The ordinate shows the plasma level; abscissa shows the time after i.p. administration of the drugs (n=8; error bars=SD).

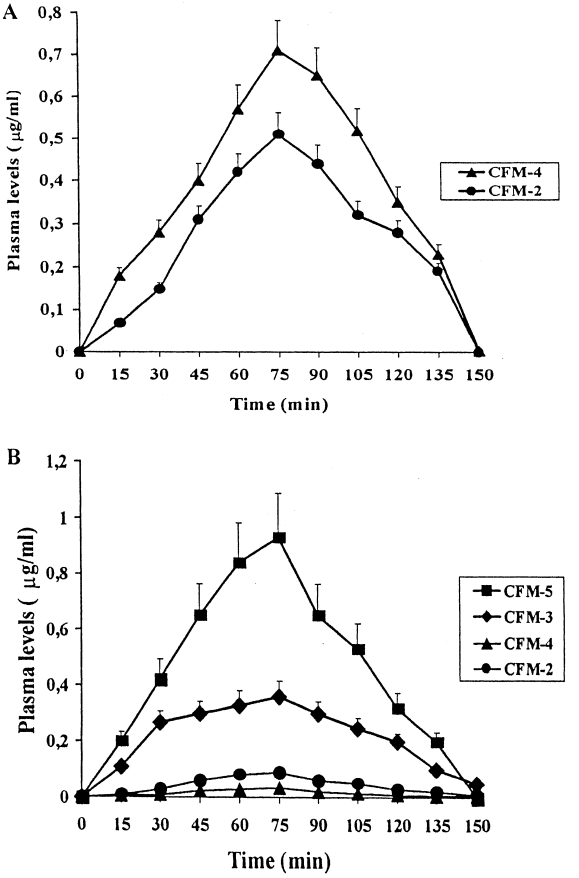


Fig. 5. Time profiles of plasma concentrations after i.p. administration in rats of CFM-4 (A) and CFM-5 (B). The ordinate shows the plasma level; abscissa shows the time after i.p. administration of the drugs (n=8; error bars=SD).

centrations of Sprague–Dawley rats treated with CFM-2 (Fig. 4A) and CFM-3 (Fig. 4B). In Fig. 4B we observe two curves: one curve was due to inoculated drug, and the other curve was due to its demethylated metabolite, CFM-2. The CFM-3 peak plasma concentration (C_{max}) was achieved 45 min after administration, whereas after 90 min plasma concentration decreased significantly. The C_{max} for CFM-2 was achieved 45 min after drug administration, after which time, the plasma level decreased very slowly. Both CFM-2 and CFM-3 were undetected in plasma 150 min after administration.

Fig. 5 shows the time profiles of plasma concentrations of Sprague–Dawley rats treated with CFM-4 (Fig. 5A) and CFM-5 (Fig. 5B). In Fig. 5A one curve was due to the inoculated drug, the other curve was due to CFM-2, obtained by nitroreduction of the 4′-substituent of the aryl ring in C-1. Peak plasma concentrations of CFM-4 and CFM-2 were achieved 75 min after administration, the plasma levels decreased significantly after 90 min.

Plasma concentration–time profiles of rats treated with CFM-5 showed four curves. In Fig. 5B one curve was due to inoculated drug; another curve was due to its demethylated metabolite, i.e., CFM-4. The other two curves were due to CFM-2 and CFM-3 obtained by nitroreduction of the 4′-substituent of the aryl ring in C-1. The C_{max} was achieved 75 min after administration, and the plasma level decreased

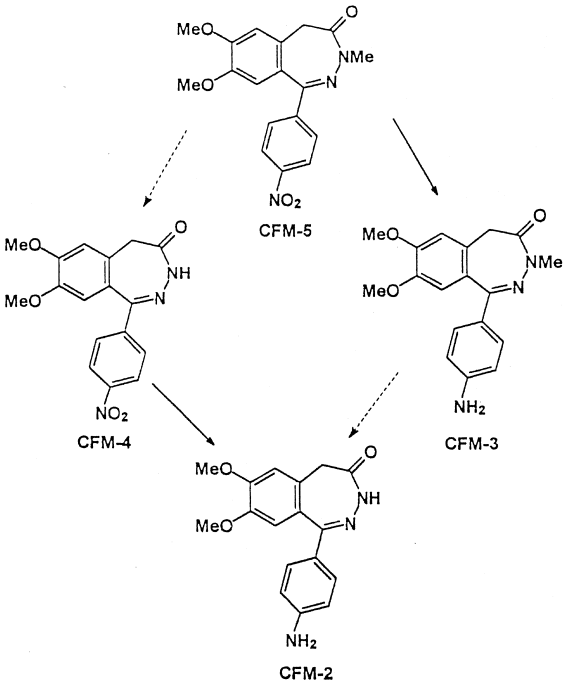


Fig. 6. Metabolic pathways of the studied 2,3-BZs in Sprague–Dawley rats.

significantly after 90 min. CFM-5 and its metabolite were not detected in plasma 150 min after administration.

Fig. 6 reports the metabolic pathways of the studied compounds in Sprague–Dawley rats, showing the possibility of nitroreduction and/or *N*-dealkylation.

4. Conclusion

This report describes the methodology and validation of a HPLC assay for the simultaneous determination of CFM-2, CFM-3, CFM-4 and CFM-5 in rat plasma. The method appeared rapid and simple.

The extraction procedures from plasma demonstrate excellent efficiency. The reversed-phase chromatography was selective. The sensitivity of the method allowed one to study the pharmacokinetics of these 2,3-BZs in Sprague–Dawley rats.

Our study suggests that the CFM-3, CFM-4 and CFM-5 are a prodrugs of CFM-2, and that they are biotransformed via different metabolic pathways.

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