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Short Communication

Separation and determination of aminohalogenbenzophenones by high-performance liquid chromatography with electrochemical detection

F. J. Rodríguez, R. M. Jiménez and R. M. Alonso

Departamento de Química Analítica, Facultad de Ciencias, Universidad del País Vasco, Apdo. 644, 48080 Bilbao (Spain)

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ABSTRACT

A high-performance liquid chromatographic method with electrochemical detection has been developed for the determination of three aminohalogenbenzophenones: 2-amino-2′,5-dichlorobenzophenone, 2-amino-5-chlorobenzophenone and 2-amino-5-bromo-2′-fluorobenzophenone, metabolites of benzodiazepinooxazoles and other psychotropic drugs. A mobile phase of methanol–water (65:35), containing 5 m*M* KH_2PO_4 appeared to be the optimal when a 4-μm, 60-Å Nova-Pak C_{18} column and a flow-rate of 0.75 ml/min (130 bar) were used. The temperature was optimized at 30°C. The amperometric detector, equipped with glassy carbon electrode, was operated at 1.3 V *versus* Ag/AgCl in the DC mode. The method was applied to the determination of these compounds at two concentration levels: ppm and ppb (ng/cm^3) using 2-amino-5-chlorobenzophenone as internal standard. The limit of determination was 750 pg/ml of biological fluid for each compound, and recoveries greater than 97% were obtained for spiked samples of urine and serum, using C_{18} Sep-Pak cartridges in the sample clean-up procedure.

INTRODUCTION

Benzodiazepinooxazoles, which are psychotropic drugs used as anxiolytic agents [1,2], have been determined by polarographic [3–5] and spectroscopic techniques [6]. These compounds, and some of their analogous benzodiazepines, are hydrolysed to aminohalogenbenzophenones, which also are metabolites of these drugs [7] and are usually determined by thin-layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC) with UV detection [8].

Most reports on electrochemical detection (ED) for HPLC have dealt with amperometry [9], because of the ease of its operation at extremely low detection limits [10].

This paper describes an HPLC–ED method for the separation and determination in biological fluids of three aminohalogenbenzophenones, 2-amino-2′,5-dichlorobenzophenone (DCB), 2-amino-5-chlorobenzophenone (MCB) and 2-amino-5-bromo-2′-fluorobenzophenone (BrFB), which are metabolites of the benzodiazepinooxazoles: cloxazolam, mexazolam, oxazolam and haloxazolam.

Correspondence to: Dr. R. M. Jiménez, Departamento de Química Analítica, Facultad de Ciencias, Universidad del País Vasco, Apdo. 644, 48080 Bilbao, Spain.

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EXPERIMENTAL

Apparatus and column

The HPLC system consisted of a Model 2150-LKB (Pharmacia, Barcelona, Spain) HPLC pump, and a Rheodyne (Pharmacia) Model 7125 injector with a loop of 20 μl.

The electrochemical detector was a PAR Model 400 with a glassy carbon cell (EG&G Princeton Applied Research, Instrumatic, Madrid, Spain). It was operated in the DC mode at +1.3 V *vs.* an Ag/AgCl electrode, with a 1-s low-pass filter time constant, and a current range between 500 and 50 nA. Chromatograms were recorded using an LKB Model 2221 integrator. The chart speed was 0.5 cm/min and the attenuation was 8 mV f.s.

The columns were (i) a 15 cm × 3.9 mm I.D. stainless-steel prepacked reversed-phase column containing 4-μm 60-Å Nova-Pak C_{18} (Waters Assoc., Barcelona, Spain) and (ii) a 30 cm × 3.9 mm I.D., 10-μm, 125-Å μBondapak C_{18} column (Waters Assoc.). The column was kept at constant temperature using a Waters temperature-control system.

Reagents and solutions

MCB and DCB were obtained from Aldrich (Bilbao, Spain) and BrFB from Lancaster Synthesis (Bischheim, France). MCB was used as the internal standard.

Stock solutions were prepared by weighing 10 mg of MCB ($C_{13}H_{10}NOCl$, MW = 231.68), DCB ($C_{13}H_9NOCl_2$, MW = 266.13) and BrFB ($C_{13}H_9NOBrF$, MW = 294.13) into a volumetric flask, and making up to 100 ml with methanol. Solvents were Lab-Scan HPLC grade, and water used was obtained by the Milli-RO and Milli-Q Waters systems. All the reagents used were Merck Suprapur (Bilbao, Spain).

Chromatographic conditions

Methanol–water (65:35, v/v) containing 5 m*M* potassium dihydrogenphosphate was used as the mobile phase. The salt served as the supporting electrolyte. This phase was filtered with a 0.45-μm membrane, and the air was removed from the phase by bubbling helium through. The Nova-Pak C_{18} column head-pressure was 130 bar at a flow-rate of 0.75 ml/min. The injection volume was 20 μl. The temperature was kept constant at 30°C.

Sample clean-up procedure

A C_{18} Sep-Pak cartridge was used to clean the biological samples. A Waters Sep-Pak C_{18} was inserted in a luer tip of a syringe and prewashed with 5 ml of methanol followed by several water washings. Then 1 ml of biological sample was forced through the cartridge with the syringe. The matrix was washed with 4 ml of Millipore water and 1 ml of methanol–water (20:80), because the aminohalogenbenzophenones studied did not elute from the cartridge until the concentration of methanol was increased to 30%, and then eluted with 2 ml of methanol. The extract was dried under vacuum. The residue was dissolved in 200 μl of mobile phase, and 20-μl aliquots were injected into the chromatograph.

Electrode maintenance

The electrode was cleaned at the end of each working day by keeping it at −600 mV for 60 s and after that at +1.2 V for 10 min. This operation was repeated three times, using a mobile phase of pure methanol at a flow-rate of 0.75 ml/min.

Also, weekly or when the signal-to-noise ratio decreased, the glassy carbon electrode was cleaned with a tissue wet with water to remove any precipitated salts, and then with a tissue soaked with methanol.

RESULTS AND DISCUSSION

In static conditions, the 1,4-benzodiazepinooxazoles cloxazolam, haloxazolam and oxazolam are not oxidized at a glassy carbon electrode. The drugs are hydrolysed in acidic and alkaline media to aminohalogenbenzophenone derivatives, which undergo a unique oxidation wave in the pH range 1–13 [11]. The oxidation peak potentials are constant at pH values lower than 3 and shift to less positive potentials as the pH increases.

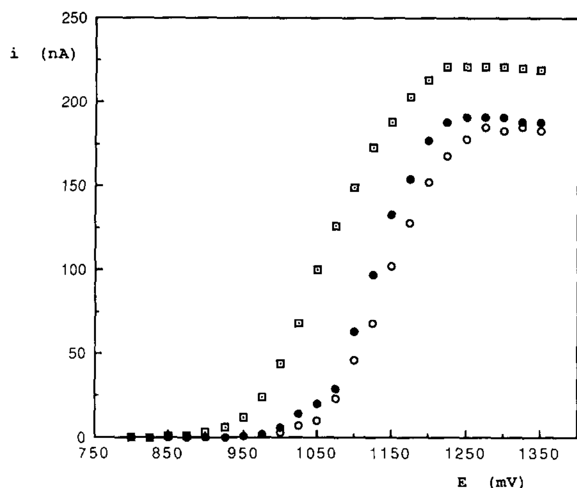

Fig. 1. Hydrodynamic voltammograms of (□) MCB, (○) DCB and (●) BrFB. Amount of solute injected: 80 ng in methanol–water (65:35) containing 5 mM KH_2PO_4.

On the basis of this information, a chromatographic system with amperometric detection can be used for the determination of these compounds.

Optimization of the chromatographic system

Hydrodynamic voltammetry of each compound was carried out to choose the optimum potential (Fig. 1). An oxidative potential of 1.3 V was chosen as the working potential because it is the lowest potential necessary for the oxidation of the three aminohalogenbenzophenones.

The neutral species is the predominant one for all three aminohalogenbenzophenones (pK_a < 1) [12], in the pH range (3–7) that is the optimum for the reversed-phase column used. For this reason, the pH of the mobile phase could not be considered as a parameter for the optimization of the retention times.

The supporting electrolyte used, which is necessary for the amperometric detection, was potassium dihydrogenphosphate. The effect of the electrolyte concentration on the signal-to-noise ratio was studied: an increase of the background signal was observed when the electrolyte concentration increased. A concentration of 5 mM was chosen as optimum.

Different ratios of methanol–water and acetonitrile–water containing 5 mM potassium dihydrogenphosphate were used as the mobile phase. The ratio 65:35 (methanol–water) was chosen as the optimum, since a good resolution in terms of k' (BrFB = 4.30; DCB = 4.71; MCB = 5.18) as well as a good elution time of monochlorobenzophenone, lower than 8 min, was achieved (Fig. 2).

As expected [13], increased temperature caused a reduction of k', which decreased the selectivity of the process without affecting the sensitivity. A temperature of 30°C was used throughout the work.

The DC and pulse modes of amperometric detection were evaluated for the three compounds studied using the system described above. The sensitivity was similar in the two modes under optimum conditions.

When optimum chromatographic conditions had been established, a quantitative method for

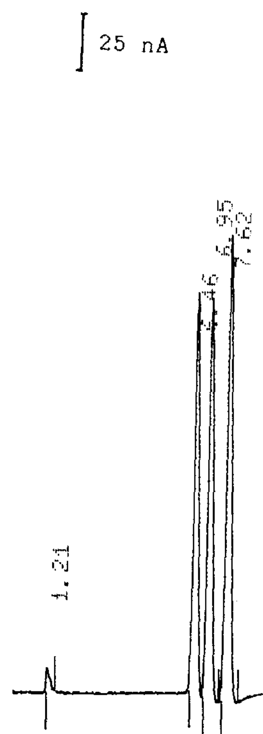

Fig. 2. Separation of MCB, DCB and BrFB, using amperometric detection and a Nova-Pak C_{18} column; mobile phase, methanol–water (65:35) containing 5 mM KH_2PO_4; flow-rate, 0.75 ml/min; oxidative potential, 1.3 V *vs.* Ag/AgCl.

TABLE I

DETERMINATION OF DCB AND BrFB USING MCB AS INTERNAL STANDARD AT TWO CONCENTRATION LEVELS: ppm AND ppb

Compound	Concentration internal standard	Linear range	Slope[a]	r^2	R.S.D. (%)	Determination limit (ppt)
BrFB	4 ppm	0.5–10 ppm	$2.78 \cdot 10^{-1}$	0.998	0.62[b]	750
	300 ppb	50–1000 ppb	$3.12 \cdot 10^{-3}$	0.996	0.76[c]	
DCB	4 ppm	0.5–10ppm	$2.87 \cdot 10^{-1}$	0.997	0.54[b]	750
	300 ppb	50–1000 ppb	$3.20 \cdot 10^{-3}$	0.997	1.50[c]	

[a] Ratio/concentration.
[b] Ten determinations at the 4-ppm level.
[c] Ten determinations at the 400-ppb level.

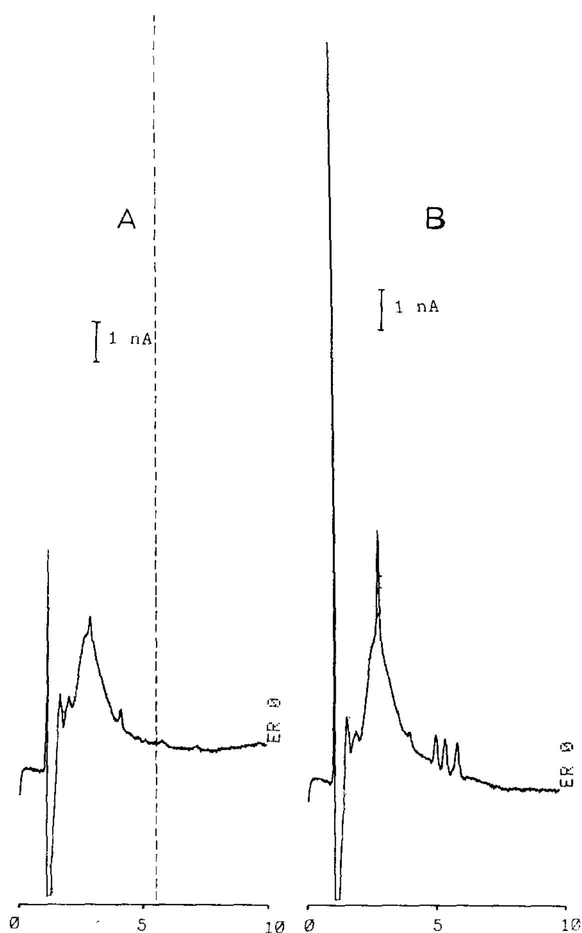

Fig. 3. Chromatograms of a blank urine extract (A) and a spiked urine extract (B), obtained after clean-up with a C_{18} Sep-Pak cartridge. Amount injected, 0.4 ng.

the simultaneous determination of DCB and BrFB, using MCB as internal standard, was developed at two concentration levels: ppm and ppb (Table I).

The limit of determination obtained for each of aminohalogenbenzophenones was 750 pg/ml of biological fluid.

Analytical applications

The low determination limits achieved have allowed the method to be applied to the quantitation of both DCB and BrFB in biological fluids (urine and serum). Fig. 3 shows typical chromatograms for blank urine and for urine samples containing the three aminohalogenbenzophenones after clean-up with Sep-Pak.

Samples of urine and serum spiked with concentrations of 40 ppb for both metabolites (urine) and 10 ppb (serum) were treated by the sample clean-up procedure previously described.

The reproducibility obtained for ten samples of urine and serum, in terms of relative standard deviations was: urine, DCB 5.80% and BrFB 4.80%; serum, DCB 7.49% and BrFB 6.51%.

Quantitative recovery was obtained for urine and serum samples at a concentration of 100 ppb with the internal standard at 300 ppb: 98.97 ± 4.20% for BFB and 97.81 ± 3.90% for DCB (mean ± S.D., n = 7). The high recovery achieved with the Sep-Pak C_{18} cartridge, in comparison with liquid–liquid extraction [14], is re-

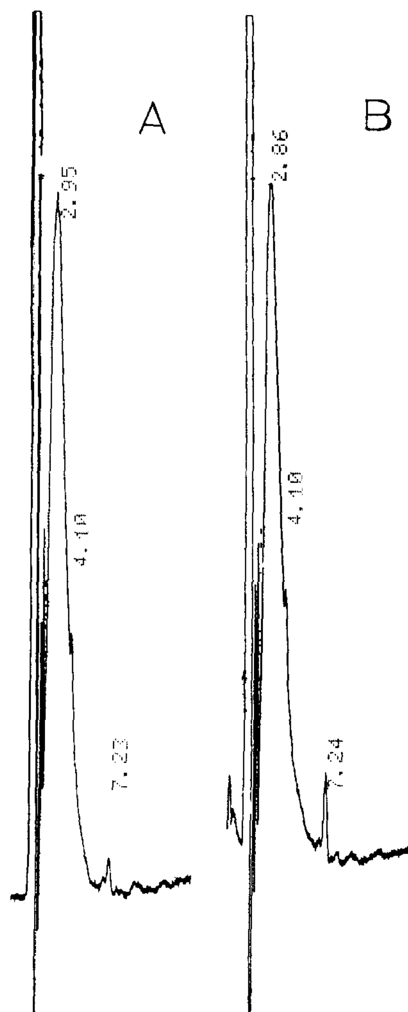

Fig. 4. Chromatograms obtained after clean-up with a C_{18} Sep-Pak cartridge of (A) a plasma sample and (B) a plasma sample after the addition of 150 ppb of DCB.

markable. Results obtained with serum were similar.

The chromatographic method developed has been applied to the analysis of DCB in plasma obtained from a Sprague–Dawley rat, 1 h after intraperitoneal injection (0.5 ml/200 g body weight) of the parent compound, cloxazolam. A plasma sample of 3.5 ml was cleaned following the sample clean-up procedure, the residue was dissolved in 1 ml of mobile phase, and 20-μl aliquots were injected into the chromatograph.

Fig. 4 shows the chromatogram corresponding to the plasma sample and the one obtained after the addition of 40 ng of DCB to 250 μl of the sample. It can be seen that the peak at 7.23 min corresponds to DCB, the metabolite of cloxazolam.

CONCLUSION

This chromatographic method, developed for the determination of aminohalogenbenzophenones, can be used for the analysis of the parent compounds, benzodiazepinooxazoles, in real samples, because they are one of their metabolites. On the other hand, HPLC–ED has been shown to be a suitable method for monitoring the hydrolysis of these benzodiazepinooxazoles, permitting the verification of the hydrolysis scheme previously reported [15], and could be used to isolate the hydrolysis products.

The use of a C_{18} Sep-Pak cartridge to clean up the samples offers as the advantages of simplicity and high recovery values over liquid–liquid extraction. ED permits limits of determination as low as 750 ppt (with a 20-μl loop), which is lower than those normally obtained by HPLC with UV detection: 50 ppb at 220 nm and 98 ppb at 254 nm.

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