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Short Communication

Validation of GC method to measure a new analgesic (E-4018) in biological samples. Comparison between HPLC and GC method*

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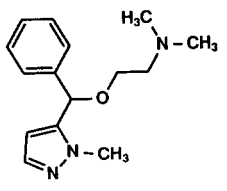
Introduction

E-4018 {1-methyl-5-[(2,*N*,*N*-dimethylamino-ethoxy) (phenyl) methyl]-1*H*-pyrazole citrate} (**I**) is a substance with considerable analgesic activity, far more potent than that of aspirin and other non-steroid, anti-inflammatory drugs. In addition, it is characterized by a complete lack of ulcerogenic effects. The studies already performed show that E-4018 does not follow any of the usual mechanisms of action, since it is neither an inhibitor of prostaglandin biosynthesis nor does it behave similarly to the opiate analgesics [1]. The aim of the study was to develop a simple and sensitive method to quantify E-4018 in plasma and urine samples from pharmacokinetic studies. Several methods have been described for the determination of diphenhydramine (**II**) in biological fluids by gas chromatography (GC) [2–4]. Due to its structural similarity to E-4018, a GC method with nitrogen–phosphorus detection, capillary column and diphenhydramine as internal standard have been used. This analytical method was compared with the results obtained by a liquid chromatography (HPLC) method that uses UV detection.



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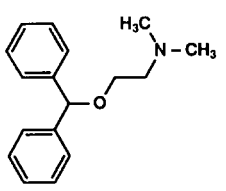

(II)

Experimental

Chemicals and reagents

The analgesic E-4018 was supplied by Laboratorios Dr Esteve, S.A. (Spain) and the internal standard, diphenhydramine hydrochloride was obtained from Sigma. Hexane, methanol and isopropanol were reagent grade, supplied by Merck. Sodium hydroxide, assessed solution, was supplied by Panreac. The organic solvent used for extraction was prepared by mixing 450 ml of hexane with 50 ml of isopropanol.

Experimental standard solutions

Stock standard solutions of E-4018 were prepared in methanol at concentrations of 2000 and 500 μg ml^{-1} (S_1) and of diphenhydramine at 2000 and 100 μg ml^{-1} (S_2). S_1 and S_2 solutions were checked by UV spectrophotometry and were stored at −20°C.

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Instrumentation

GC method. Analyses were performed using a Hewlett–Packard 5890 gas chromatograph equipped with a 7673A automatic sampler, a nitrogen–phosphorus detector and a 3390A integrator. A methyl silicone capillary column, 300 × 0.25 mm i.d. with 0.25 μm film thickness, (SPB-5, Supelco), was used. The injector (splitless mode) and detector were operated at 280°C. Oven temperature was programmed from 130 to 280°C (for plasma samples) or 265°C (for urine samples) at a rate of 10°C min^{-1}. Helium was used as a carrier gas at a flow rate of 1.2 ml min^{-1}. Air and hydrogen flow rates were 80 and 3 ml min^{-1}, respectively.

Retention times for E-4018 and diphenhydramine were 12.8 and 12.6 min, respectively.

HPLC method. For the HPLC assay the following instruments were used: a Waters Associates model 501 pump, a Waters Associates UV 441 detector, set to 229 nm, a Hewlett–Packard 1050 automatic liquid sampler, a Shimadzu C-R3A integrator.

The separation was carried out using a 150 × 4 mm column packed with 10-μm CN-micro-Bondapak (Waters Associates, Milford, MA, USA). The mobile phase was 1/60 M potassium phosphate (pH 6.0)–methanol (90:10, v/v) with a flow rate of 1 ml min^{-1}. Retention times for E-4018 were 10–12 min and for diphenhydramine, 15–16 min.

Extraction procedure for plasma and urine samples

GC method. Aliquots (500 μl) of plasma (250 μl of urine) were placed in extraction tubes. A 1 μg mass of the internal standard (1.25 μg for urine), 200 μl of 1 N NaOH (100 μl for urine) and 4 ml of hexane–isopropanol mixture were added, shaken mechanically for 20 min and centrifuged at 3000 rpm and 4°C for 15 min. The organic phase was transferred to conical tubes and brought to dryness under N_2 stream at 40°C. The dry residue was redissolved with 100 μl of hexane and 2 or 3 μl were injected into the chromatograph. Representative chromatograms are shown in [Fig. 1(A,C)].

HPLC method. The extraction was done by adding to 500 μl of plasma, 1 μg of internal standard, 500 μl of 1 N NaOH and 10 ml of chloroform. The dry residue was redissolved with 100 μl of mobile phase and 20 μl were injected into the chromatograph. Representative chromatograms are presented in [Fig. 1(B,D)].

Calibration procedure

Standard curves of E-4018 in plasma samples were prepared at the following concentrations: 0.05, 0.1, 0.2, 0.5, 1, 5, 10 and 20 μg ml^{-1}.

Standard curves of E-4018 in urine samples were prepared at two different concentration ranges: 0.5, 5, 20 and 40 μg ml^{-1} and 50, 100, 200 and 400 μg ml^{-1}, in order to quantify samples with a high E-4018 concentration.

For the pharmacokinetic study, analytical quality control (QC) samples were prepared, from separately weighed stock solutions, by another analyst, and the concentration of these samples was unknown to the analyst responsible for the study.

The standard and QC samples were aliquoted (0.7 ml per tube) immediately after spiking and stored at −20°C until needed.

Results and Discussion

Linearity

The linearity of the method was tested in order to demonstrate a proportional relationship of response versus analyte concentration over the working range.

Peak area ratios between E-4018 and the internal standard versus E-4018 concentrations were subjected to least-squares regression analysis.

The linearity for the GC method within the concentration range 0.05–20 μg ml^{-1} was $y = 0.474x + 0.128$ ($r = 0.998$). For the HPLC method, within the range 0.2–5 μg ml^{-1} was $y = 0.918–0.026$ ($r = 0.989$).

The limits of detection based on signal to noise ratio of approximately 2 to 1 were 0.1 ng (injected on the column) for the GC method and 10 ng for the HPLC method.

The limits of quantitation defined as the lowest concentration that can be determined from the plasma with a RSD <15% were about 30 and 200 μg ml^{-1} for the GC and HPLC methods, respectively.

Precision and accuracy

Intra-day precision of the GC was determined by measuring five plasma samples at

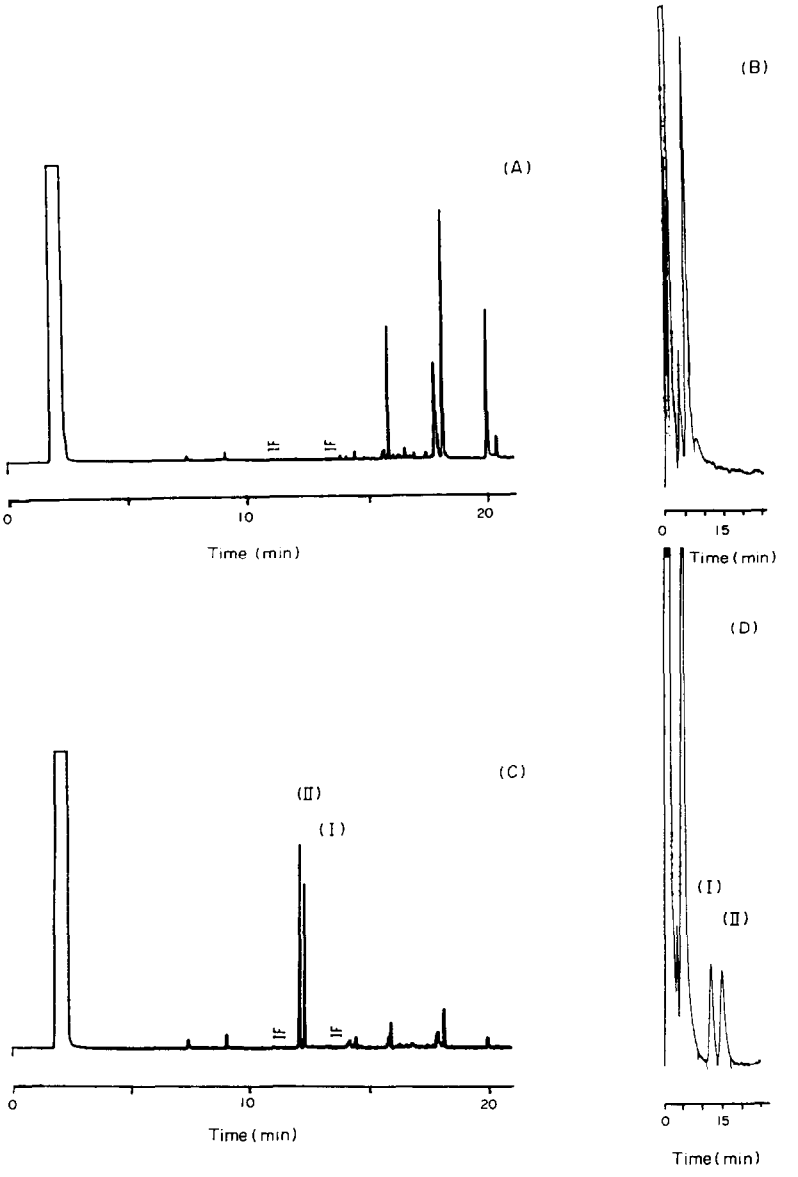

Figure 1
Representative chromatograms of E-4018 analysis (A) and (B) correspond to GC and HPLC drug-free dog plasma samples; (C) and (D) correspond to GC and HPLC plasma sample obtained from a Beagle dog 1.5 h after oral administration of 12.5 mg kg^{-1} E-4018 (1 μg ml^{-1}).

three different concentrations: 0.05, 0.5 and 10 μg ml^{-1}. The RSD of the peak areas were: 14.2, 5.8 and 1.0%, respectively.

Inter-day precision measurement for GC was 7.67, 4.27 and 3.95% at 0.2, 1 and 10 μg ml^{-1}, respectively (n = 20). The inter-day precision for HPLC was 14.8, 19.4 and 13.7% at 0.2, 1 and 5 μg ml^{-1}, respectively.

The accuracy of the GC and HPLC methods was evaluated at three different concentrations. The results are shown in Table 1.

Specificity

Drug-free plasma samples were collected from 24 Beagle dogs and surveyed for interferences by GC method. No interference peaks were seen in 21 samples. A small interference peak representing less than 10% of the lowest standard value of the retention time of E-4018 was observed in three samples of plasma.

Recovery

Recovery for the GC method was calculated

Table 1
Precision and accuracy of the method for determination of E-4018 in dog plasma

Inter-day precision					
GC method: Concentration ($\mu g\ ml^{-1}$)	n	RSD (%)	HPLC method: Concentration ($\mu g\ ml^{-1}$)	n	RSD (%)
0.2	20	7.7	0.2	11	14.8
1	20	4.3	1	8	19.4
10	20	3.9	5	8	13.7

Intra-day precision					
GC method: Concentration ($\mu g\ ml^{-1}$)	n	RSD (%)	HPLC method: Concentration ($\mu g\ ml^{-1}$)	n	RSD (%)
0.05	5	14.2	0.2	5	11.7
0.50	5	5.8	0.5	5	8.2
10	5	1.0	5	5	11.2

Accuracy					
GC method: Real value	Mean values observed ($\mu g\ ml^{-1}$)	Mean relative error (%)	HPLC method: Real value	Mean values observed ($\mu g\ ml^{-1}$)	Mean relative error (%)
0.2	0.19	−7.3	0.5	0.48	−3.7
1	1.02	1.6	1	0.98	−2.0
10	10.00	0.0	5	5.36	7.2

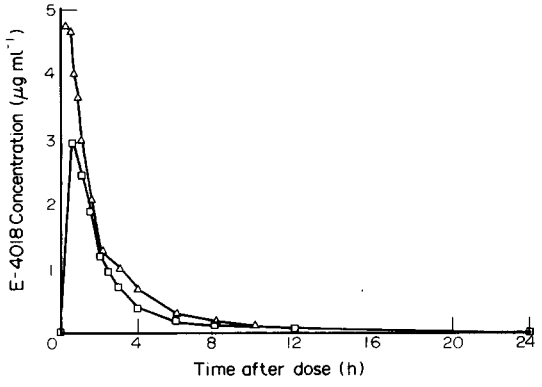

Figure 2
Mean concentrations of E-4018 in plasma samples from 12 Beagle dogs following single oral and intravenous doses of 12.5 mg kg^{-1}.

by comparing extracted standard samples with unextracted standards, which represented 100% recovery. The recovery of E-4018 was 85.6% (RSD = 7.6%) with concentration range 0.05–10 $\mu g\ ml^{-1}$.

Pharmacokinetic study

In a pharmacokinetic study with 12 Beagle dogs more than 500 plasma and urine test samples were measured, together with calibration and control samples. Quality control samples (n = 20) analysed concurrently with the test samples were in good agreement with concentrations added (MSE < 4%). Figure 2 illustrates mean plasma concentrations of E-4018 following single oral and intravenous doses of 12.5 mg kg^{-1}.

A sensitive, selective and rapid GC method has been developed for the determination of E-4018, a structural analogue of diphenhydramine, in plasma and urine. After a single extraction step, the method shows good accuracy and precision and is robust for routine work.

The method was used for a pharmacokinetic study without any problems in a series of more than 650 chromatograms.

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