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On-line fully automated solid-phase extraction–liquid chromatography analysis of 1,2-dihydro-4-(1,2-dihydro-2-oxo-1-pyridyl)-2,2-dimethyl-1-oxonaphthalene-6-carbonitrile (UR-8225), a new potassium channel opener, in plasma samples☆

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Abstract

A fully automated reversed-phase HPLC method for pharmacokinetic studies was developed for the determination in plasma samples of 1,2-dihydro-4-(1,2-dihydro-2-oxo-1-pyridyl)-2,2-dimethyl-1-oxonaphthalene-6-carbonitrile (compound I), a new potassium channel opener. On-line solid-phase extraction was performed with disposable C_{18} cartridges. After clean up, the samples were eluted and transferred onto an RP-18 analytical column, where separation was performed with a mobile phase of acetonitrile–10 m*M* di-*n*-butylamine phosphate (28:72, v/v). Ultraviolet absorbance detection was used at 236 nm. The detector response was linear in the range 25–2500 ng/ml, and the lowest limit of quantitation was determined at 2.5 ng/ml. The inter-day variability was $<4\%$ for samples at 1000 ng/ml and $<15\%$ for samples at 10 ng/ml. This method was used for the pharmacokinetic study of compound I in rats at three different dosage levels.

1. Introduction

The last few years have seen an increase in the synthesis and development of new chemical compounds with potassium channel opening activity. These compounds relax smooth muscle through a mechanism which involves an outflow of intracellular potassium and subsequent hyperpolarization of the membrane potential. This process results in a decrease in the intracellular calcium concentration and relaxation of the cell. Given the pharmacological activity of potassium channel openers, it has been suggested that these compounds may be very effective in cardiovascular therapy as antihypertensives, coronary vasodilators, anti-ischemics or cardioprotectors. The compound [1,2-dihydro-4-(1,2-dihydro-2-oxo-1-pyridyl)-2,2-dimethyl-1-oxonaphthalene-6-carbonitrile] (compound I, see Fig. 1) is a new K^+ channel opener sensitive to glybenclamide [1,2] and with demonstrated vasodilator and hypertensive activity in animals [3,4].

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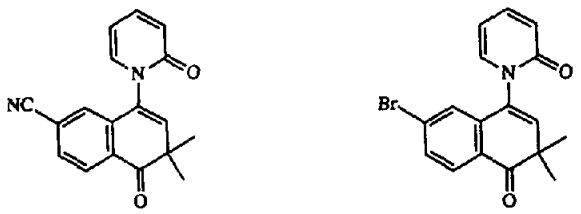

I II

Fig. 1. Structures of I and II (internal standard).

Due to the high pharmacological activity of I, therapeutic doses will necessarily be low, producing low plasma concentrations and making the quantitation of the drug in plasma samples difficult. Until now, the pharmacokinetics of I has been unknown, since no method has been published which would allow its measurement in plasma. Our aim was to develop an analytical method for determining plasma concentrations of I with high precision and accuracy as well as with a low limit of quantitation. This method would be valid for pharmacokinetic studies in preclinical and clinical research during drug development.

This paper describes a fully automated method for the determination of I in plasma, based on solid-phase extraction (SPE) with disposable C_{18} cartridges and on-line separation on the HPLC analytical column. While off-line procedures, performed by liquid–liquid extraction, solid-phase extraction or protein precipitation, allow injection of a part of the final extract, on-line procedures allow utilization of the total amount of analyte extracted. In this study the method was applied in a preclinical trial in rats.

2. Experimental

2.1. Chemicals

The standards of I and [6-bromo-1,2-dihydro-4 - (1,2 - dihydro - 2 - oxo - 1 - pyridyl) - 2,2 - dimethylnaphthalene-1-one] (compound II, internal standard (I.S.), see Fig. 1) were supplied by J. Uriach y Cia. (Barcelona, Spain).

For the mobile phase, an acetonitrile gradient grade from SDS (Solvants Documentation Syntheses, Peypin, France) and reagent D-4 from Waters-Millipore (Millford, MA, USA) were used. Reagent D4 is an ion-pairing agent consisting of a concentrate of di-*n*-butylamine phosphate, which on dilution in 1 l of water gives a 10 m*M* solution at pH 3. The use of this mobile phase additive results in an improvement in the peak shapes.

The methanol used was Lichrosolv gradient grade from Merck (Darmstadt, Germany). The water was deionized and purified through a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

The method uses the Prospekt automated sample preparation system (Spark Holland, Emmen, Netherlands). Fig. 2 shows a schematic representation of the system, which was described for the first time by Nielen et al. [5]. This system consists of three units: the Marathon automatic injector, the solvent delivery unit (SDU) and the Prospekt unit. The Marathon automatic injector has a rotary valve which loads the untreated sample into a 500-μl loop and injects it onto the SPE cartridge. The solvent delivery unit (SDU) is equipped with an exchange flow valve for six solvents and with a low-pressure pump. The SDU supplies the required solvents both for transferring the samples from the loop to the SPE cartridge and for performing clean-up procedures. The SPE cartridge is automatically changed after each sample in the Prospekt unit, which contains a rotary valve to connect the cartridge on-line with the analytical column so that all the analyte retained in the cartridge is eluted by the mobile phase of the HPLC system. V2 and V3 are auxiliary valves which are used in our application only to determine the recovery in the SPE cartridge by direct injection of standards onto the analytical column. This unit also contains the software for programming the automated sample preparation process.

The HPLC system consisted of two ABI 400 high-pressure pumps (Applied Biosystems, Foster City, CA, USA), an ABI 491 high-pressure

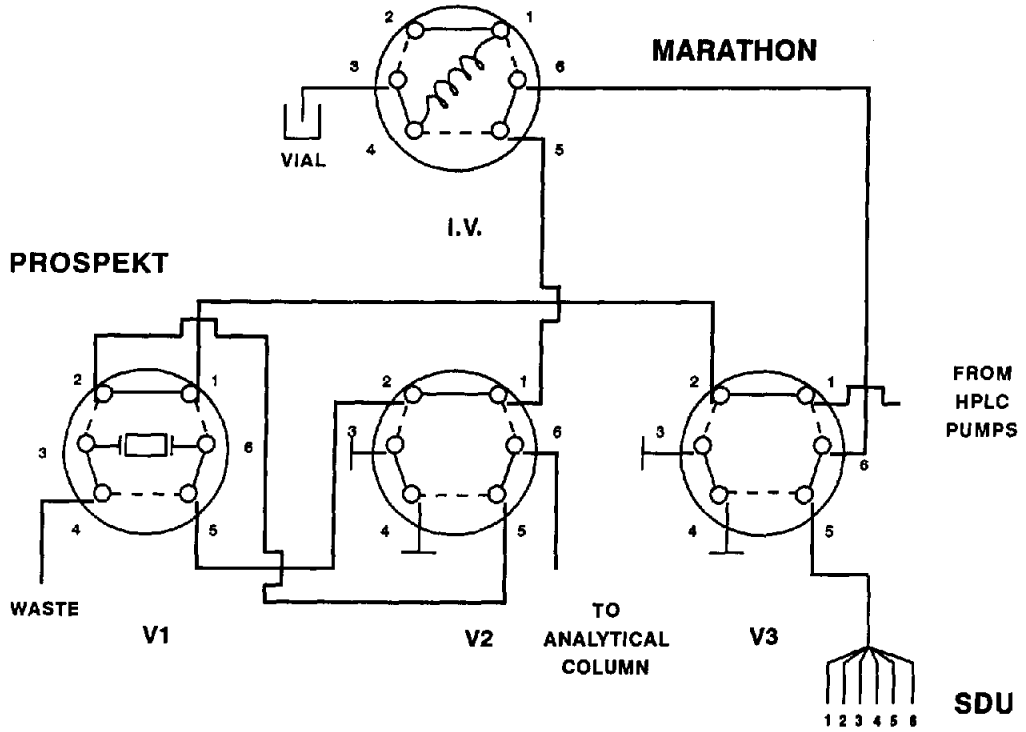

Fig. 2. Diagram of valves of the Prospekt system (SDU, Prospekt and Marathon). For automated sample preparation, V2 and V3 should be permanently in position ———, and in position – – – for direct injection. I.V. directs the solvents from the SDU through the loop (position – – –) or the bypass (position ———). V1 bypasses (position ———) or does not bypass (position – – –) the flow in the mobile phase through the SPE cartridge.

dynamic mixer (Applied Biosystems), and an ABI 1000S diode-array detector (Applied Biosystems). For data collection and processing PE Nelson 900 Series interface and Nelson Model 2600 chromatographics software were used (Perkin-Elmer Nelson Analytical, Cupertino, CA, USA).

2.3. Chromatographic conditions

The analytical column used was a Brownlee Spheri-5 RP-18 100 × 4.6 mm I.D., 5 μm particle size (Applied Biosystems, San Jose, CA, USA). The precolumn used was a Brownlee New Guard RP-18 (15 × 4.2 mm I.D., 7 μm particle size). The mobile phase was degassed by vacuum filtration of the separate components and consisted of 28% acetonitrile and 72% reagent D-4 (10 m*M* di-*n*-butylamine phosphate, pH 3) mixed at high pressure; elution was isocratic at 1.0 ml/min. Detection was performed by UV absorption at 236 mm.

2.4. Sample preparation

SPE was performed in cylindrical cartridges (10 × 2 mm I.D., Spark-Holland) packed with 20 mg of 40-μm octadecyl silica (Baker, Deventer, Netherlands). Plasma samples (1 ml) were mixed with 50 μl of internal standard solution (I.S.) in methanol, introduced into the vials and placed in the Marathon injector. A 500-μl aliquot of sample was loaded into the loop and drawn towards the SPE cartridge by 3 ml of water. When all of the sample had been transferred from the loop to the SPE cartridge, clean up was continued with 1.5 ml water (flow-rate 1.5 ml/min). Finally, by switching rotatory valve V1 (Fig. 2), the cartridge was connected and eluted

Table 1
Sample preparation program in Prospekt unit

Time (min)	Solvent (SDU)	SDU flow-rate (ml/min)	Switch VI	Switch V1	Comment
00:00	1		Load	Purge	Position valves. Change the cartridge. Load the sample from vial into loop
00.01		1.5			Activate cartridge with methanol.
00:30	2				
00:31		1.5			Condition cartridge with water
02:30			Inject		Load sample from loop onto cartridge.
04.30			Load		Sample clean up with water
05.30				Elute	Cartridge on-line with HPLC.

on-line with the analytical column for 2 min. The procedure is summarized in Table 1.

2.5. Samples

For method validation, rat plasma samples were used spiked with known amounts of I to give final concentrations of 2–2500 ng/ml. Pharmacokinetic study of I was carried out in rats after oral and intravenous administration at doses of 1.0, 0.5 and 0.1 mg/kg. Blood samples were taken by cardiac puncture at 0.083, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 24.0 h after administration, using EDTA as anticoagulant. The plasma fraction was separated by centrifugation for 15 min at 2000 *g*. All samples were analysed immediately after being taken or were kept at −20°C until analysis. Four rats were used for every sampling time.

3. Results and discussion

Fig. 3 shows the chromatograms of a blank plasma sample (A), a spiked plasma sample (1000 ng/ml) (B), and a real sample (C).

3.1. SPE recovery

The peak areas of I and II were measured in chromatograms obtained after direct injection (without prior extraction) of the compounds dissolved in mobile phase. These areas were compared with those obtained in the chromatograms of plasma samples treated by on-line SPE to which the same amount of analyte had been added. The recoveries of I and II from plasma were ca. 90% and 95%, respectively. Table 2 shows the results for the different concentrations of I in plasma samples. The recovery of II was only studied at a concentration of 500 ng/ml. No variations in recovery were observed when buffers with different pH (pH range 3–8) were used as solvents for clean up. Nor was any improvement observed in sample clean up as compared with that obtained using water. There was a slight improvement in the sample clean up with 5% ethanol in water, but in this case the recovery of I was reduced to 80% in samples at 1000 ng/ml. Since our aim was to develop a method for pharmacokinetic purposes and were therefore seeking the lowest quantitation limit, this reduction in SPE performance was not acceptable.

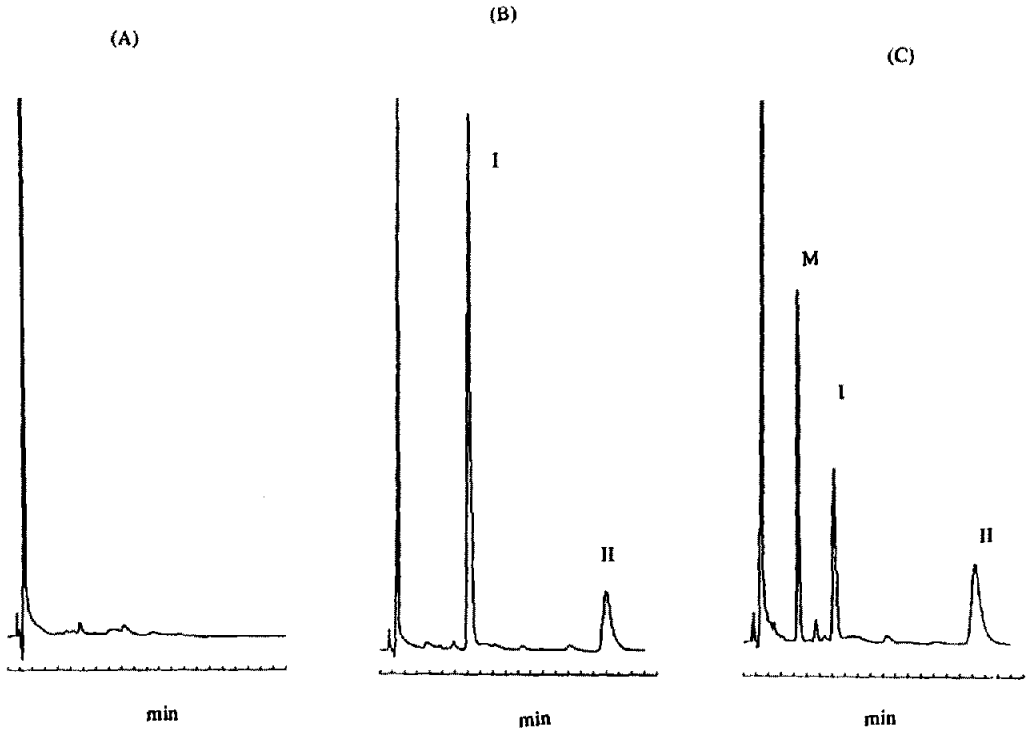

Fig. 3. Chromatograms corresponding to (A) blank plasma, (B) plasma sample spiked with 1000 ng/ml of I, and (C) a real sample. In the real sample (C) a new peak (M) appears, corresponding to a possible metabolite of I, still unidentified.

3.2. Linearity

Linearity was studied by analysing plasma standards in the range 25–2500 ng/ml. The regression line obtained was $y = [-1.6 \cdot 10^{-2}(\pm 5.2 \cdot 10^{-2})] + [3.13 \cdot 10^{-3} \pm 4.62 \cdot 10^{-5}]x$, with a correlation coefficient of $r = 0.9998$. The intercept of the regression line is close to zero, within the intercept confidence interval.

Table 2
Recovery of I comparing peak area of I in SPE extracted spiked plasma samples with peak area of I in standards directly injected (without extraction) in mobile phase solution

Concentration (ng/ml)	Recovery (mean ± S.D.) ($n = 3$)
2500	97.6 ± 0.7
1000	92.5 ± 0.6
500	88.4 ± 1.0
250	90.2 ± 1.7
25	83.0 ± 1.4
10	97.9 ± 6.1
5	103.0 ± 11.7

Each spiked plasma sample is compared with the mean of peak areas of 4 standards of I in mobile phase solution.

3.3. Lower limit of quantitation

The lower limit of quantitation (LLQ) was determined according to the definition of Kucharczyc [6], who proposes the LLQ to be considered as: "The lower limit of quantitation is that concentration of the analyte in the matrix of interest for which the confidence interval at 95% probability does not overlap with the confidence interval of the matrix blank standard". For this purpose a regression line was established with plasma standards of 2, 5, 10 and 20 ng/ml ($r = 0.997$), and the confidence interval was calculated in order to determine the LLQ. This gave a result of 2.5 ng/ml.

3.4. Precision and accuracy

To estimate the precision and accuracy, repeated analyses were made of plasma samples to which known quantities of I had been added. The final concentrations were: 2500, 1000, 200, 40, 25, 10 and 2 ng/ml. Samples of 1000 and 10 ng/ml were also repeatedly analysed on two other days. The calibrations were made with plasma standards of 2000 and 100 ng/ml for the samples of 2500, 1000, 200 and 40 ng/ml, and of 20 and 5 ng/ml for the samples of 25, 10 and 2 ng/ml. The standards were treated in the same way as the samples. According to Shah et al. [7], the precision (C.V.) established in the validation of the analytical methods for bioavailability, bioequivalence and pharmacokinetic studies is 15%. The same authors established that a variation in accuracy of ± 15% is admissible, except for the lower limit of quantitation, for which the acceptable variation in both precision and accuracy is set at 20%. The results of the intra-day and inter-day trials, given in Table 3, confirm that the C.V. and accuracy obtained for this method are valid for pharmacokinetic purposes. Samples of 2 ng/ml which are below the LLQ present an acceptable C.V. but the variation in

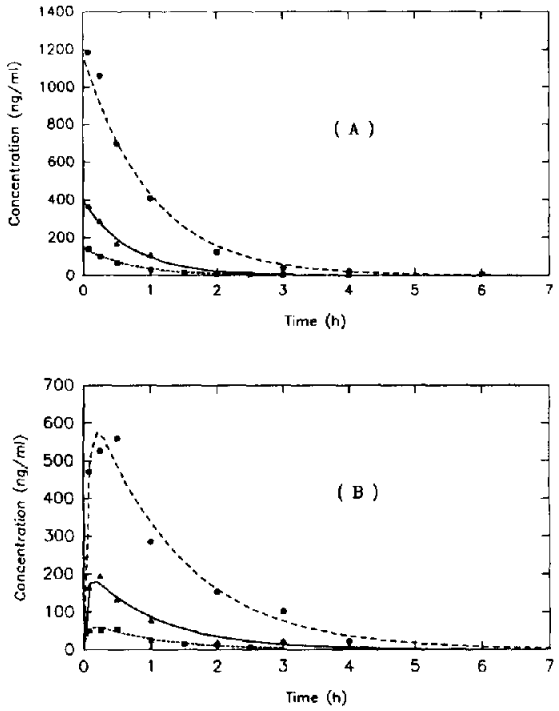

Fig. 4. Plasma levels of I after (A) i.v. administration and (B) oral administration, at doses of 1.00 mg/kg (●), 0.25 mg/kg (▲) and 0.10 mg/kg (■). The principal pharmacokinetic values are $t_{1/2} = 50$ min, V = 997 ml/kg, Cl = 837 ml/kg h, and absolute bioavailability = 75%.

Table 3
Precision and accuracy of the determination of I in spiked plasma samples

Concentration (ng/ml)	*n*	Mean (ng/ml)	S.D. (ng/ml)	C.V (%)	Accuracy (%)
Intra-day					
2500	5	2514	37	1.5	100.6
1000	7	1024	28	2.7	102.4
200	5	217	5	2.4	108.5
40	5	36	4	10.8	90.0
25	5	24	2	6.9	97.1
10	6	11	2	14.0	108.7
2	5	3	1	19.2	124.5
Inter-day					
1000	20	1021	40	3.9	102.1
10	20	9.8	1	14.7	98.4

accuracy is slightly higher than the accepted 20%.

3.5. Practical application

The method described has been successfully used for the analysis of more than 700 rat plasma samples for pharmacokinetic purposes, achieving good reproducibility. A significant saving in time has been achieved over that which would have been spent on liquid–liquid extraction. In rats a possible metabolite of I has been detected, which still has to be identified.

This method has been applied in the pharmacokinetic study of I in rats at three different dosage levels (1.0, 0.25 and 0.1 mg/kg) allowing the following pharmacokinetic parameters to be determined: biological half-life, 50 min; clearance, 837 ml/h kg; volume of distribution, 997 ml/kg; bioavailability, 75%; t_{max}, between 15 and 30 min; and C_{max}, 575, 183 and 60 ng/ml for doses of 1.0, 0.25 and 0.1 mg/kg, respectively. Fig. 4 shows the pharmacokinetic profile of I after i.v. and oral administration at the three different doses in Sprague–Dawley rats. New results of studies not yet published, indicate that the present method would be valid for studying the pharmacokinetics of I in humans and other animal species, and also for use in clinical trials.

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