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Validated liquid chromatographic method for the determination of N-3-(2,2,5,5-tetramethyl-3-pirrolin-3-carboxamidopropylphthalimide hydrochloride), a novel antiarrhythmic agent in human plasma

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Abstract

A simple high-performance liquid chromatographic method with ultraviolet absorbance detection has been developed to determine the concentration of N-3-(2,2,5,5-tetramethyl-3-pirrolin-3-carboxamidopropylphthalimide hydrochloride; A-2545), a new antiarrhythmic agent from human plasma. Separation of the investigated compound and internal standard was achieved on a Nucleosil 7 C_{18} column with a 0.01-*M* potassium dihydrogenphosphate buffer (pH 2.5)–methanol (60:40, v/v) mobile phase. The detection was performed at 220 nm. During the determinations, buspirone served as the internal standard. The compounds were isolated from plasma on a Bakerbond C_{18} solid-phase extraction cartridge and the mean absolute recovery was 92.9%. The limit of quantitation was found to be 10 ng/ml. The bioanalytical method was validated with respect to linearity, within- and between-day accuracy and precision, system suitability and stability. All validated parameters were found to be within the internationally required limits. The developed analytical method for A-2545 was found to be suitable for application in pharmacokinetic studies and for human drug monitoring. © 1998 Elsevier Science B.V.

Keywords: A-2545; Tetramethylpirrolincarboxamidopropylphthalimide hydrochloride; Buspirone

1. Introduction

The compound code-numbered A-2545 [N-3-(2,2,5,5-tetramethyl-3-pirrolin-3-carboxamidopropyl-phthalimide hydrochloride)] is a new phthalimide derivative, synthesised by ICN Alkaloida (Tiszavasvári, Hungary). Pharmacologic effects of the compound were scrutinised in detail by the Pharmacological Institute of Szent-Györgyi University of Medicine (Szeged, Hungary).

An excellent antiarrhythmic effect of the drug-proband has been proved in standard animal models. Its efficacy in preventing ventricular and supraventricular fibrillation is more pronounced following both oral and intravenous administration than that of chinidine, lidocaine or mexiletine. At the same time, the new compound also has a wider therapeutic range and also possesses a pronounced and long-lasting cardioprotective effect. The drug-proband slightly reduces myocardial contractility and arterial blood pressure.

According to its electrophysiological properties, the drug-proband can be classified as belonging to the Vaughan–Williams I/B class [1].

As far as we know, high-performance liquid chromatographic (HPLC) methods have not been developed for the quantitative determination of A-2545 in human plasma to date.

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This assay was designed in order to quantify A-2545 in human plasma after A-2545 was administered orally for human phase I and II clinical trials. A basic requirement was to ensure that a plasma concentration of 10 ng/ml could be determined (limit of quantification, LOQ) of A-2545.

During the determinations, buspirone was used as the internal standard.

2. Experimental

2.1. Materials

A-2545 and buspirone (Bus) as the internal standard (I.S.) [8-[4-(4-(2-pyrimidinyl)-1-piperazinyl)-butyl]-8-azospiro[4,5]decane-7,9-dione] were provided by ICN Alkaloida. The structural formulae for these compounds are depicted in Fig. 1.

Methanol, acetonitrile (gradient grade) and ethylamine (70%) purchased from Merck (Darmstadt, Germany). Bakerbond C_{18} cartridges (1 ml) were from J.T. Baker (Phillipsburg, NJ, USA). Potassium dihydrogenphosphate and EDTA disodium salt were obtained from Reanal (Budapest, Hungary). All chemicals used were of analytical grade.

2.2. Chromatographic conditions

For liquid chromatography, an LC-6A pump, an SPD-6A detector and a C-R6A integrator were used (Shimadzu, Kyoto, Japan). For sample injection, a SIL-6B (equipped with a SCL-6B system controller; both from Shimadzu) autosampler fitted with a 50-μl loop was applied.

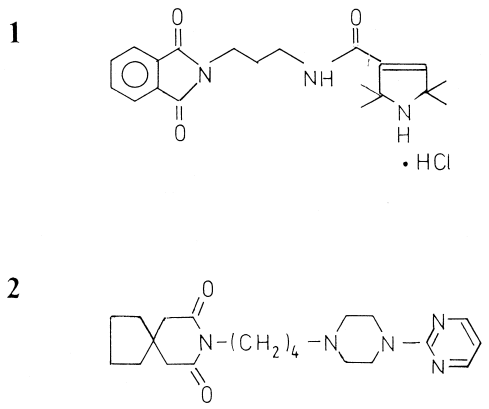

Fig. 1. Structural formulae for A-2545 (1) and the internal standard (buspirone) (2).

The separation was accomplished at ambient temperature (air-conditioned room with a temperature of 22±2°C), on a Nucleosil 7 C_{18} (250×4 mm I.D.) analytical column equipped with a Nucleosil 7 C_{18} (30×4 mm I.D.) guard column (Bio-Separation Technologies, Budapest, Hungary). The flow-rate was 1.0 ml/min. The detection wavelength was 220 nm. The mobile phase consisted of 0.01 *M* potassium dihydrogenphosphate buffer (pH 2.5)–methanol (60:40, v/v). The eluent was filtered through a 0.45-μm nylon 66 membrane (Supelco, Bellefonte, PA, USA) and degassed by ultrasonication.

2.3. Solutions

A 1 mg/ml stock solution of A-2545 was prepared using a mixture of methanol–water (1:1, v/v). Dilutions for the working solutions were also made with 50% methanol (100, 10 and 1 μg/ml).

The I.S. (Bus) stock solution (0.25 mg/ml) and the working solutions (2.5 μg/ml) were also made using 50% methanol.

When stored at −20°C, both stock solutions were stable for at least eight weeks. The working solutions were freshly prepared each week and stored in a refrigerator at 4°C.

Blank human plasma was prepared from blood obtained by venipuncture from the cubital vein. Disodium-EDTA was used as an anticoagulant at a concentration of 1 mg/ml of whole blood. The anticoagulant was dissolved in distilled water (100 mg/ml) and 0.1 ml of the solution was added to 10 ml of blood. Whole blood supplemented with the anticoagulant was centrifuged at 1500 *g* for 10 min and the resulting plasma was stored at −20°C until processing.

2.4. Sample processing

To 1 ml of sample, 250 ng of the I.S. (100 μl of a 2.5 μg/ml solution in 50% methanol) and 1 ml of 50 m*M* potassium dihydrogenphosphate solution, pH 7.2, were added. After homogenising the mixture by vortex-mixing, the sample was transferred to a

Bakerbond C_{18} solid-phase extraction (SPE) cartridge (1 ml) that had been activated previously with 2×1 ml of methanol and with 2×1 ml of 50 m*M* potassium dihydrogenphosphate buffer, pH 7.2. After sample application, the cartridge was washed with 2 ml of 50 m*M* potassium dihydrogen phosphate buffer, pH 7.2 and 0.5 ml of 50% methanol, before being dried by sucking air through the cartridge. After 15 min, the solute was eluted with 1 ml of acetonitrile–ethylamine (99:1, v/v). The eluate was evaporated to dryness under a stream of nitrogen at 37°C. The dry residue was dissolved by vortex-mixing it in 250 μl of the mobile phase and 50 μl aliquots were injected onto the column.

For calibration, ''spiked'' model samples were made from pooled drug-free plasma by adding the actual amount of working solution together with I.S.

2.5. Method validation

This method was validated according to internationally accepted criteria [2–4].

2.5.1. Quality control (QC) samples

For method validation, QC samples were prepared from pooled human plasma, in advance, at 10, 50, 250 and 1000 ng/ml A-2545 levels. QC samples were stored frozen at −20°C in 1 ml aliquots. Each QC sample was fortified with the I.S. (250 ng to 1 ml of plasma) just prior to sample processing.

2.5.2. Precision of the chromatography system

Both compounds were injected five times from their own working solutions (1 μg/ml of A-2545 and 2.5 μg/ml of the I.S.).

2.5.3. Summary of calibration curve parameters

Calibration samples were prepared by adding 0, 10, 50, 100, 250, 500 and 1000 ng of A-2545 to 1 ml of blank pooled human plasma. Each sample was fortified with 250 ng of the I.S. The samples were processed and chromatographed as described above. Six parallel determinations were made at each concentration level.

To construct the calibration curve, peak area ratios of the A-2545 and of the I.S. were plotted against nominal concentration values using the KALIB computer program. The calibration curve was fitted to the measured points by the weighted least squares method using a weighting factor, $w=1/y^2$. The *F* test for linearity and linear regression analysis were chosen for testing linearity.

2.5.4. Within-day precision and accuracy

The within-day precision and accuracy of this method were determined by analysis of QC samples at four different concentration levels. Six parallel determinations were made at each level.

2.5.5. Between-day precision and accuracy

The between-day precision and accuracy were determined by the analysis of QC samples at four different concentration levels on six different days covering a four-week study period.

2.5.6. Determination of absolute recovery

Blank plasma samples (1 ml) spiked with 10, 50, 100, 250, 500 and 1000 ng of A-2545 were processed without the addition of I.S. by the SPE procedure. The I.S. (250 ng) was added only to the evaporation residue of the eluate dissolved in the mobile phase. Three parallel determinations were made at each concentration. The peak area ratios obtained with the extracted samples were compared to those of the corresponding aqueous solutions of A-2545 and the I.S.

2.5.7. Stability test

The stability of A-2545 in human plasma was studied at 50 and 1000 ng/ml levels after two and four weeks of storage at −20°C. The samples were fortified with the I.S. just prior to processing. Three parallel determinations were made at each concentration and for both storage periods.

The stability of QC plasma samples during three freeze/thaw cycles was determined. Triplicate QC samples, at concentrations of 50 and 1000 ng/ml, were frozen for 24 h at −20°C. The samples were thawed at room temperature for 2 h and subsequently returned to the freezer for 24 h. The samples were thawed, processed and injected. The mean measured concentrations of the stability samples were compared to the nominal concentrations.

The stability of the extracted and dissolved 50 ng/ml samples in the autosampler were measured by comparing the ratios of the original standard line to

the ratios of the standard line injected 0, 6, 12, 24 and 36 h later.

3. Results and discussion

An HPLC method using UV detection (220 nm) was developed for the quantitative determination of A-2545 in human plasma.

Using a Nucleosil 7 C_{18} stationary phase and the binary mobile phase of 0.01 *M* potassium dihydrogenphosphate buffer (pH 2.5) and methanol (60:40, v/v) provided an almost symmetrical peak shape for both the compound to be determined and the I.S.

Fig. 2 shows a chromatogram of a blank plasma extract of a spiked (250 ng of the I.S.) plasma extract obtained after processing the samples using a Bakerbond C_{18} (1 ml) SPE cartridge. Fig. 3. shows a typical chromatogram of a volunteer's plasma sample after oral administration of 50 mg of A-2545. It can be clearly seen from the chromatograms that no endogenous peak interferes with the chromatographic peak of A-2545 and we could measure not only the parent drug (A-2545) but also a putative metabolite (t_R ca. 58 min). Considering the kinetic behaviour, this peak was assumed to be a metabolite.

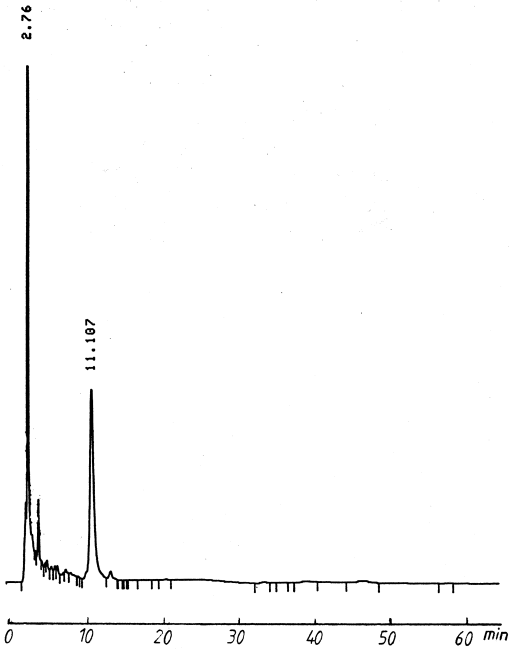

Fig. 2. Chromatogram of a characteristic blank plasma extract spiked with 250 ng of the I.S. The retention time of 11.107 min corresponds to the I.S. (buspirone). Chromatographic conditions are described in the text.

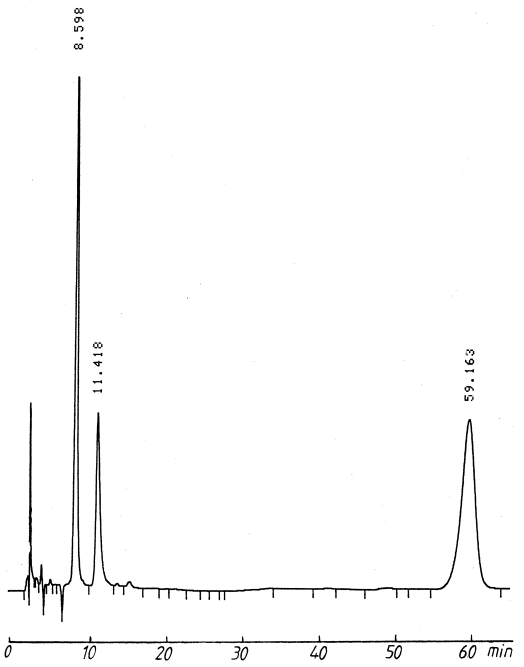

Fig. 3. Representative chromatogram of a human plasma extract of a volunteer 3 h after oral administration of 50 mg of A-2545. Retention times of 8.598, 11.418 and 59.163 min correspond to A-2545, I.S. (buspirone) and a tentative metabolite, respectively. Chromatographic conditions are described in the text.

Based on the reproducibility of the chromatographic conditions, the mean retention time for A-2545 was 8.58 min, while the same parameter for the I.S. was 11.41 min. On the basis of five parallel determinations, the reproducibility [R.S.D. (%)] of the retention time was 0.11% for A-2545 and 0.14% for the I.S., whereas the reproducibilities of the peak area values were 1.72 and 1.67% for A-2545 and the I.S., respectively. Detailed validation data are presented in Tables 1–3.

The reproducibility of the method (R.S.D.) is

Table 1
Summary of the calibration curve parameters

Nominal concentration (ng/ml)	A-2545/I.S. peak area ratio (mean±S.D.)	R.S.D. (%)	Measured concentration (ng/ml) (mean±S.D.)	*n*
10	0.199±0.032	16.08	10.04±0.09	6
50	0.775±0.122	15.74	49.54±1.66	6
100	1.500±0.095	6.35	99.26±4.24	6
250	3.711±0.298	8.03	250.89±12.21	6
500	7.371±0.339	4.59	501.90±18.35	6
1000	14.721±2.187	14.85	1005.92±50.20	6

Equation for the calibration curve: $y=0.0526+0.01458x$, $r=0.9999$.

within the accepted limit of 20% for the analysis of biological samples. On the basis of the validation parameters, the limit of quantitation (LOQ) achieved with this method was 10 ng/ml of plasma and the limit of detection was 5 ng/ml.

The F test for linearity ($F_{4,30}=0.9584$) indicated that the calibration curve obtained by the regression model with $w=1/y^2$ was good. The equation of the calibration curve covering the 10 to 1000 ng/ml A-2545 plasma concentrations was $y=0.0526+0.01458x$ ($r=0.9999$), where y stands for A-2545/I.S. ratio and x denotes the plasma concentration of A-2545 (Table 1).

The R.S.D. values of within- and between-day precision, determined by the analysis of QC samples, were always lower than 10% for all investigated concentrations, except one (the LOQ), and the corresponding accuracy values never exceeded 9% (Table 2).

The absolute recovery of A-2545 was 92.9%, on average, and the dependence of recovery on concentration was negligible.

The stability tests indicated that, in stock solutions, both A-2545 and the I.S. were stable for up to eight weeks. As regards the stability of A-2545 in human plasma, no significant decomposition was observed after either two or four weeks of storage at −20°C. The accuracy of A-2545 in the plasma after storage was between 95.7 and 107.0% (Table 3).

The date in Table 3 indicate that freeze/thawed samples remained stable under the applied conditions.

Table 2
Precision

Nominal concentration (ng/ml)	Measured concentration (ng/ml) (mean±S.D.)	R.S.D. (%)	Accuracy (%)	*n*
Within-day precision and accuracy of the method				
10	10.14±0.10	9.86	101.0	6
50	49.20±1.71	3.45	98.4	6
250	253.51±11.22	4.42	101.4	6
1000	1087.0±50.90	4.68	108.7	6
Between-day precision and accuracy of the method				
10	10.91±1.90	17.43	109.0	6
50	53.52±4.80	8.97	107.1	6
250	248.31±21.12	8.49	99.3	6
1000	1008.63±53.25	5.27	100.9	6

Table 3
Stability studies

Results of stability test (at −20°C)					
Storage (weeks)	Nominal concentration (ng/ml)	Measured concentration (ng/ml) (mean±S.D.)	R.S.D. (%)	Accuracy (%)	n
2	50	52.37±4.41	8.40	104.7	3
	1000	985.61±50.90	5.16	98.6	3
4	50	53.51±5.11	9.53	107.0	3
	1000	956.75±57.23	5.98	95.7	3

Stability data for A-2545 in human plasma following three freeze/thaw cycles

Nominal concentration (ng/ml)	Measured concentrations (ng/ml) (mean±S.D.)			*n*
	Cycle I	Cycle II	Cycle III	
50	51.58±5.03	52.71±4.12	51.74±5.50	2
1000	998.82±72.26	1091.56±82.33	1070.4±77.35	2

Stability data for A-2545 in human plasma following 36 h at room temperature

Time (h)	(Nominal concentration, 50 ng/ml) Measured concentration (ng/ml)	Accuracy (%)
0	49.82	99.64
6	52.91	105.82
12	53.72	107.44
24	54.98	109.96
36	53.14	106.28

Results from the study on the stability of A-2545 in human plasma extract stored at room temperature in an autosampler for 36 h are presented in Table 3. No significant decrease in the concentrations was detected.

According to the validation results, the method is suitable for the determination of A-2545 in human plasma. This method was used for pharmacokinetic studies and for monitoring plasma levels following the oral administration of A-2545 in the course of a human phase I and II study.

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