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QUANTITATIVE DETERMINATION OF ANGIPUR DRUG SUBSTANCE IN BLOOD PLASMA BY HPLC WITH FLUORESCENCE DETECTION

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A quantitative determination method using HPLC with fluorescence detection for the xanthine derivative 3-methyl-8-(piperazin-1-yl)-7-(thietan-3-yl)-1-ethyl-1*H*-purine-2,6(3*H*,7*H*)-dione hydrochloride (Angipur drug substance) in donor blood plasma was developed and validated. The optimal conditions for quantitative determination of Angipur drug substance were KH_2PO_4 buffer (50 mM, pH 6.5); aqueous-to-organic phase ratio 55:45 v/v; added sodium heptanesulfonate modifier (0.15%); temperature 40ºC; detector extinction wavelength 290 nm; and emission wavelength 340 nm. The method sensitivity (detection limit) for Angipur was 2.5 ng/mL; limit of quantitation, 5 ng/mL. The average measurement error was ≤15%.

Keywords: HPLC, 3-methyl-8-(piperazin-1-yl)-7-(thietan-3-yl)-1-ethyl-1*H*-purine-2,6(3*H*,7*H*)-dione hydrochloride, quantitative determination.

Antiaggregant agents play an important role in prevention of thrombus formation [1, 2]. Glycoprotein IIb/IIIa receptor blockers for intravenous administration are currently the most potent antiaggregants for treating adverse outcomes in patients with acute coronary syndrome and angioplasty patients with this diagnosis and for percutaneous coronary intervention with coronary artery stents. However, this drug class causes serious side effects, the most dangerous of which are severe thrombocytopenia and hemorrhage [3]. Therefore, the search, study, and design of new domestic synthetic glycoprotein IIb/IIIa receptor inhibitors that block the final pathway of platelet aggregation with diminished side effects is an important task for improving the prevention of thrombotic conditions.

A literature analysis suggested that heterocyclic structures can inhibit platelet aggregation [4 – 6]. A new xanthine derivative, Angipur, was discovered in this compound class and could suppress platelet aggregation in *in vitro* and *in vivo* tests. Also, it was capable of binding directly to glycoprotein IIb/IIIa receptors through integrins CD41a and CD61, which was determined using cytofluorimetric analysis.

Pharmacokinetic studies are one of the most important stages in working on this problem. The obtained results enabled the factors responsible for the medicinal effect of this compound, e.g., the optimal administration mode and ranges of effective concentrations, to be evaluated [7, 8]. Physicochemical analytical methods that would allow small volumes

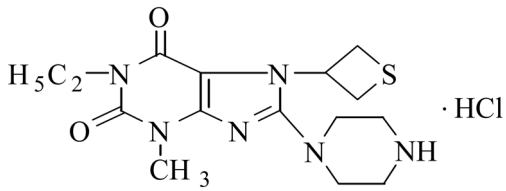

Fig. 1. Structural formula of 3-methyl-8-(piperazin-1-yl)-7-(thietan-3-yl)-1-ethyl-1*H*-purine-2,6(3*H*,7*H*)-dione hydrochloride, Angipur drug substance.

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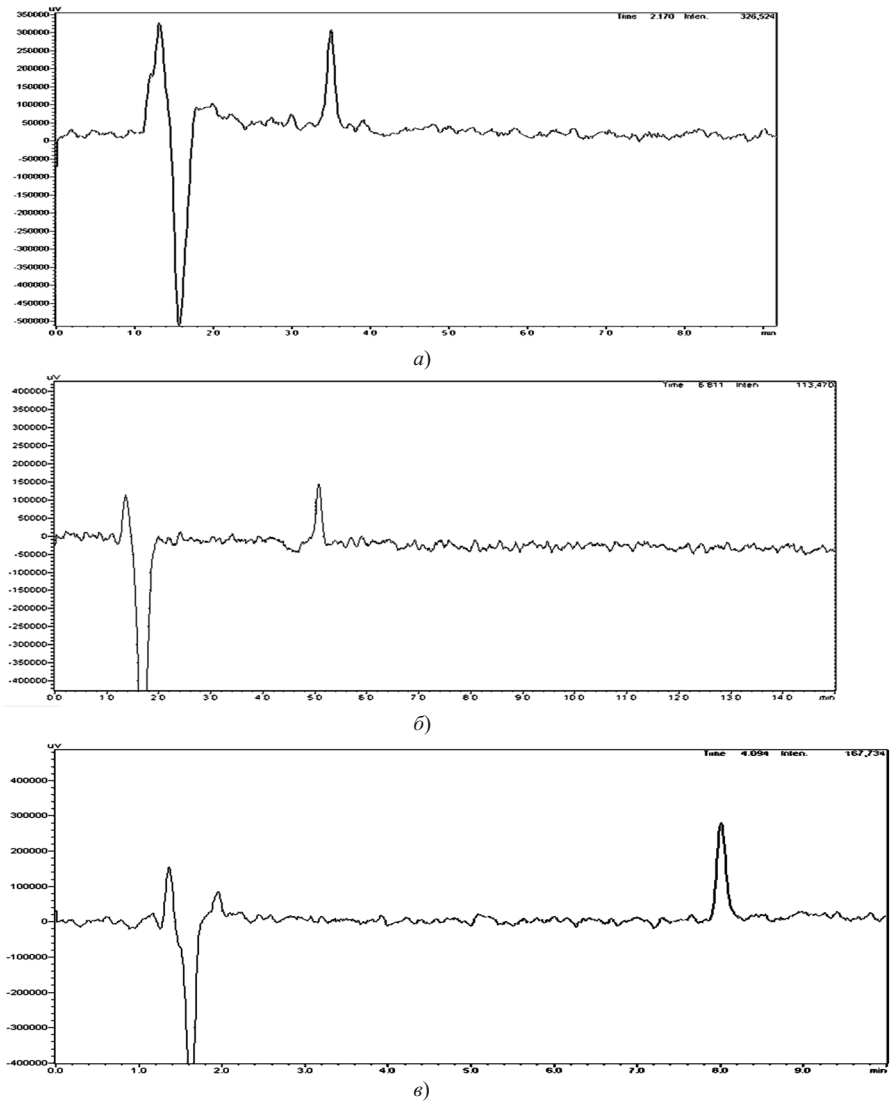

Fig. 2. Chromatogram of Angipur drug substance in aqueous solution without added modifier (*a*), with sodium dodecyl sulfate modifier (*b*), with sodium heptanesulfonate modifier (*c*). Notations: along the abscissa, time (min); along the ordinate, emission units.

of biological samples to be studied with sufficient sensitivity, specificity, precision, and accuracy are needed to conduct pharmacokinetic studies. High-performance liquid chromatography (HPLC), which is currently one of the main methods for conducting pharmacokinetic studies, satisfies these requirements.

EXPERIMENTAL CHEMICAL PART

Angipur drug substance, 3-methyl-8-(piperazin-1-yl)-7-(thietan-3-yl)-1-ethyl-1*H*-purine-2,6(3*H*,7*H*)-dione hydrochloride (Fig. 1), was used for the studies and was synthesized in the Department of Pharmaceutical Chemistry, Bashkir State Medical University, Ministry of Health of Russia (Ufa, Russia) [9].

Angipur is a white crystalline powder that is readily soluble in H_2O, poorly soluble in MeOH, and practically insoluble in Me_2CO. It decomposes upon melting.

The PMR spectrum of a solution (5%) of the substance in D_2O taken on an instrument at operating frequency 500 MHz for ^1H showed resonances with chemical shifts, intensities, and spin–spin coupling constants (δ, ppm) of 1.02 (t, 3H, *J* 7.1 Hz); 3.23 (pseudo t, 2H, *J* 8.9 Hz); 3.29 (s, 3H); 3.34 – 3.41 (m, 8H); 3.78 (q, 2H, *J* 7.1 Hz); 3.99 (pseudo t, 2H, *J* 9.3 Hz); 5.55 (pseudo pent, 1H, *J* 9.2 Hz), and a resonance at 4.76 ppm belonging to the solvent.

The IR spectrum in the range 4000 – 400 cm^{-1} taken from a KBr pellet of the substance had absorption bands corresponding to the spectrum of a reference standard of the substance.

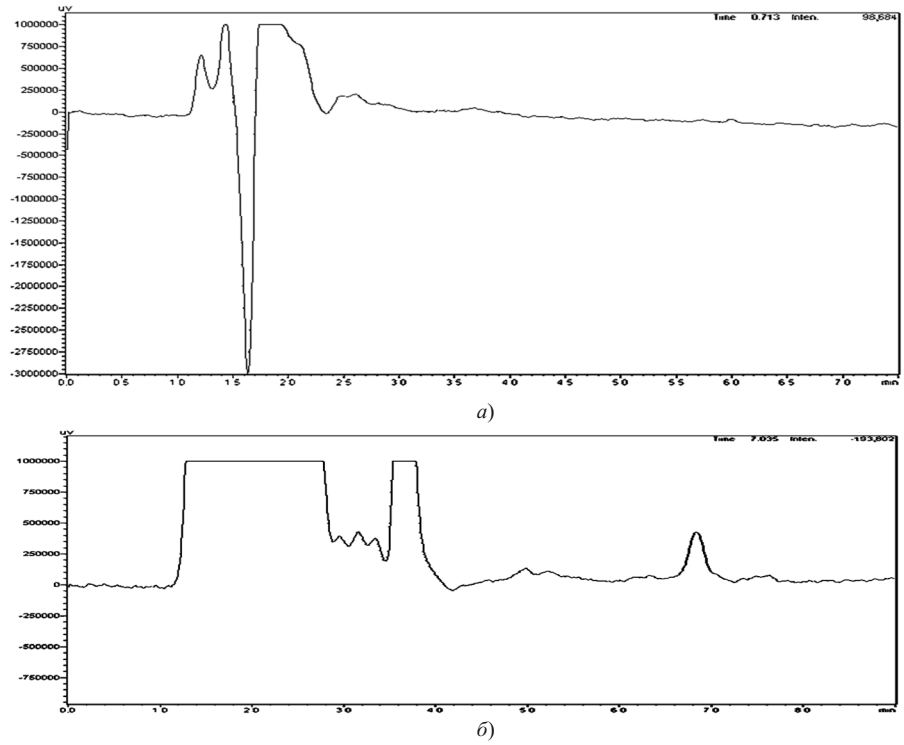

Fig. 3. HPLC chromatogram of biological matrix (blood plasma) background (*a*), Angipur drug substance in biological matrix (blood plasma) (*b*). Notations: along the abscissa, time (min); along the ordinate, emission units.

An HPLC method with fluorescence detection was developed for quantitative determination of Angipur drug substance and could determine it in a biological matrix (rat blood plasma). A liquid chromatograph (Shimadzu, Japan) was used in the work. Angipur drug substance was determined using a fluorescence detector and SUPELCOSIL LC-18 column (5 μm; 100 × 4.6 mm) and a phase modifier. The mobile phase was prepared using MeCN (UF210, Russia) and KH_2PO_4 buffer (50 mM).

EXPERIMENTAL BIOLOGICAL PART

The quantitative determination method was developed using donor blood plasma obtained at Volgograd Regional Blood Center. Blood plasma was thawed at room temperature and used as the solvent for preparing calibration solutions.

The dependence of peak areas on drug substance concentration was analyzed by regression analysis. Results were statistically processed using the Microsoft Excel computer program.

RESULTS AND DISCUSSION

Angipur drug substance was detected using extinction wavelength 290 nm and emission wavelength 340 nm.

Buffer systems in the pH range 2.5 – 7.0 were used to develop the method. The qualitative (retention time) and quantitative characteristics (chromatographic peak width, symmetry, and height) in the analysis of the compound were not significantly affected if the pH was varied.

The temperature regime was selected in the range from 4 to 60°C. The column became unstable without changing the method sensitivity if the temperature was decreased. Variations were insignificant in the range 40 – 60°C so that a temperature of 40°C was finally selected.

Aqueous-to-organic phase ratios (vol%) of 80:20, 70:30, 60:40, 50:50, 55:45, and 40:60 were used. The selectivity decreased and the sensitivity increased if the fraction of the organic phase was increased. The optimal ratio of aqueous-to-organic phases was 55:45 vol%. It gave sufficient sensitivity and separation of the analyte peak from the biological matrix background.

Mobile-phase modifiers of sodium heptanesulfonate and sodium dodecyl sulfate in various percent concentrations were used in the work.

Use of phase modifiers enabled the compound to be retained on the column with retention times providing method selectivity without decreasing the sensitivity (Fig. 2*b* and 2c). The best analytical characteristics were achieved by using sodium heptanesulfonate (0.15%).

Possible extractants for isolating Angipur from blood plasma were EtOH, MeCN, trifluoroacetic acid (TFA) (10, 30, 50, and 70%), and conc. HCl.

Samples were agitated for 20 min in an ultrasonic bath to precipitate proteins and centrifuged for 15 min at 10,000 rpm

TABLE 1. Analytical Parameters of Quantitative Determination Method for Angipur Drug Substance in Blood Plasma in the Linear Range of the Dependence of Chromatographic Peak Area on Solution Concentration

Concentration, ng/mL	Average peak area, mAu·min	Average concentration recalculated from curve, ng/mL ($M \pm m$)	Precision, %	Accuracy, ±Δ%	Average measurement error, %
2.5	19.79	2.07 ± 4.6	221.86	– 82.8	17.2
5	20.46	5.01 ± 1.15	22.99	– 100.2	0.2
10	22.06	12.19 ± 0.74	6.03	– 121.9	21.9
25	26.29	26.69 ± 1.25	4.69	– 105.16	5.16
50	32.81	50.98 ± 1.81	3.54	101.96	1.69
100	44.82	95.69 ± 3.2	3.35	95.69	4.31
500	153.59	500.66 ± 19.41	3.88	100.13	0.13

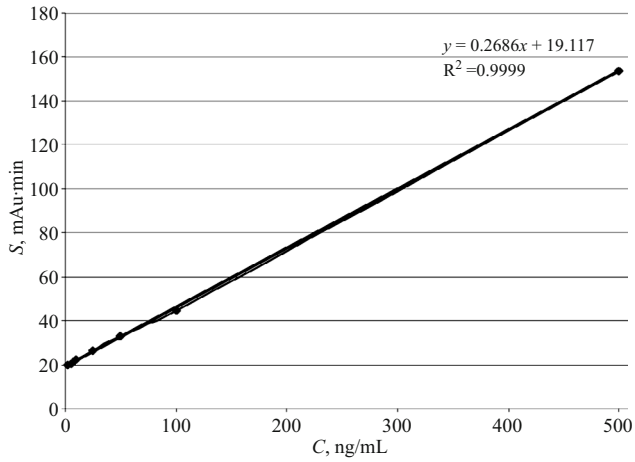

Fig. 4. Dependence of chromatographic peak area on Angipur drug substance concentration. Notations: along the abscissa, chromatographic peak area, mAU·min; along the ordinate, Angipur drug substance concentration, ng/mL.

in an Eppendorf centrifuge. The supernatant liquid was removed and injected into the injector loop (20 μL).

Use of 70% TFA gave the best extraction (100 ± 3%).

Thus, the optimal conditions for quantitative determination of Angipur drug substance were KH_2PO_4 buffer (50 mM, pH 6.5); aqueous-to-organic phase ratio 55:45 vol%; added sodium heptanesulfonate modifier (0.15%); temperature regime 40°C; extinction wavelength 290 nm; emission wavelength 340 nm; flow rate 1 mL/min; and pressure in the chromatographic system 12 MPa. The retention time was 6.9 – 7.2 min under these conditions (Figs. 2c and 3b).

The quantitative determination used concentrations in the range from 5 to 500 ng/mL. The resulting calibration curves were linear with a regression coefficient (R^2) of 0.999 (Fig. 4).

The intraday percent variations (method repeatability) were determined and were ≤ 20% in the studied concentration ranges. The interday percent variations (method reproducibility) for the compound were mainly ≤ 15% (Table 1).

The average absolute percent variations for a repeated analysis after storing the aqueous solutions for 72 h fell within the same limits, demonstrating that the analyzed substance was stable.

Freeze–thaw cycles were studied and showed that the average absolute percent variations for Angipur drug substance fell within the same limits so that it was determined to be stable under the influence of these factors.

The sensitivity of the method (detection limit) for Angipur drug substance was 2.5 ng/mL; limit of quantitation, 5 ng/mL. The average measurement error was ≤ 15%.

Thus, the quantitative determination method for Angipur drug substance had sufficient sensitivity and selectivity for solving the stated problems. The optimal extraction method was chosen and had practically no effect on the average measurement error of the chromatographic method for quantitative determination.

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