ANALYSIS OF FUROSEMIDE AND ITS MAIN METABOLITES IN BIOLOGICAL FLUIDS BY SCANNING ACCORDING TO THE FLUORESCENCE OF THIN-LAYER CHROMATOGRAMS

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Furosemide (lazix, I) is one of the most widely used diuretics. The pharmacokinetics and metabolism of I in humans have been rather fully studied [1]. Various methods have been used to determine the drug in biological fluids: colorimetric [2], fluorometric [3-6], radioisotope using [^{35}S]-furosemide [7], and chromatographic methods — gas–liquid chromatography [8] and highly efficient liquid chromatography [9]. The spectral methods are insufficiently specific and permit a determination only of the summary concentration of I and its metabolites. The known chromatographic methods presuppose a complex preliminary treatment of the samples.

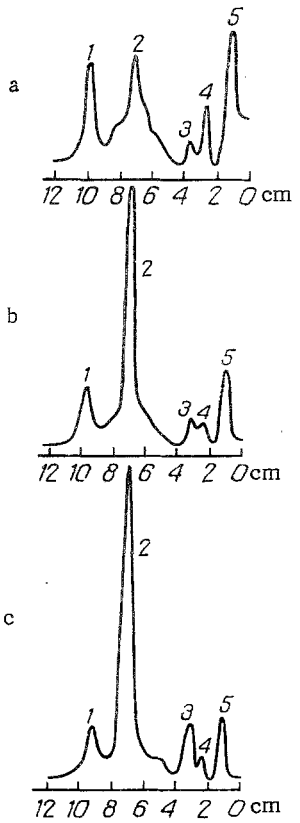

Fig. 1

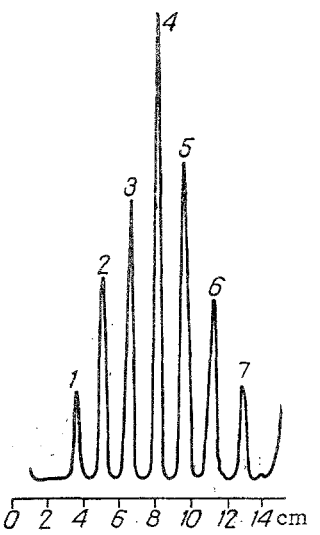

Fig. 2

Fig. 1. Densitogram of a TLC plate with applied protein-free filtrate of blood serum of a patient who took 40 mg of I internally in a single dose. Recording of the transmission $\lambda > 390$ nm, excitation at different wavelengths: a) at 320 nm; c) at 273 nm. Scanning along the path of movement of the solvent system; 1) Anthranilic acid (II); 2) furosemide (I); 3) 4-chloro-5-sulfanil-anthranilic acid (III); 4, 5) endogenous blood serum substances.

Fig. 2. Densitogram of TLC (according to I) with urine samples of a patient, containing various amounts of the drug, applied to the plate. 1-7) 10, 30, 50, 100, 60, 20, and 10 ng in a spot, respectively. Scanning was performed perpendicular to the direction of movement of the solvent system.

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An analysis of the existing methods allows us to agree with the assertion of [10], that up to the present time there is no simple method for determining I and its metabolites with sufficient sensitivity, selectivity, and high analytical reproducibility.

In a number of studies methods using thin-layer chromatography (TLC) have been proposed for the investigation of the pharmacokinetics of I. However, these methods are not without shortcomings, which do not permit their simplicity to be fully realized. Before chromatography, the biological samples are extracted with an organic solvent, and the extract is concentrated under vacuum [11-13]. In [10] and [11], the plates are sprayed with solutions of the reagents before densitometry; in [12, 13] the spots are cut out and eluted. Most of these methods are designed for the analysis only of the drug itself, and they do not permit the determination of metabolites.

We have developed a new, simple, and rapid method of simultaneous determination of I and its two main metabolites in blood serum (plasma) and urine. The method does not require preliminary treatment of the samples of biological fluids, with the exception of precipitation of the blood serum proteins with methanol. Essence of the method: the blood serum (plasma) sample after removal of proteins or the urine sample is applied on a plate, developed, and dried, after which it is scanned in a fluorescence system, recording the transmission. In the system chloroform–methanol–acetic acid (89:6:5), I and its two main metabolites — anthranilic acid (II) and 4-chloro-5-sulfanilanthranilic acid (III) — are well separated in chromatography on Silufol, being thereby separated from endogenous fluorescent substances (Fig. 1). The maximum light transmission (summary, with wavelength $\lambda > 390$ nm), and, consequently, the maximum sensitivity of fluorescent detection of spots are observed in scanning of the chromatograms irradiated with light with a wavelength corresponding to the maxima in the spectra of excitation of the substances to be analyzed: for I 279 nm, for II 320 nm, and for III 273 nm (see Fig. 1). In this case it proved convenient to scan the chromatograms not along the path of movement of the developing solvent system but perpendicular to this direction, detecting I, II, and III by three successive scans, using the optimum length of excitation each time. Consequently, if standard calibration mixtures I, II, and III and the samples to be analyzed are applied on one plate, then complete information (including calibration), necessary for an investigation of the dynamics of the concentration of the preparation and metabolites in one patient (Fig. 2), is obtained in each of three recordings. On one standard plate with dimensions 15 × 15 cm, three to four standard calibration mixtures (I + II + III) of different concentrations and five to six test samples can be applied. As a result, for a complete analysis of the dynamics of the excretion of I and its metabolites II and III with the urine in one patient (five to six time intervals of urine collection), only 1-1.5 h is required — the time necessary for the application of ten points on the plate, development of the plate, and three scans at each of three wavelengths (~2 min each). Two analyses of the dynamics of the concentrations of the drug and metabolites in the blood serum require more time for precipitation of the proteins with methanol.

Sharp symmetrical peaks are recorded on the densitogram. The absolute sensitivity of the recording at a 10:1 signal to noise ratio is 0.5-1 ng per spot. When biological fluid (50 ml) is used for the analysis, the sensitivity of the method is consequently 10-20 ng in 1 ml of sample.

The area under the peak (F) corresponding on the densitogram to a chromatographic spot proves to be strictly proportional to the amount of the substance in this spot (G) within a range of two orders of magnitude (1-100 ng) — on a plot of log E versus log G, the points lie on a straight line with a slope equal to one (Fig. 3). The method is characterized by high accuracy and reproducibility. At a concentration of I of 0.1-0.5 μg/ml, the average error in the determination of the concentration in the blood is 4-6%, and in the urine 3-5%; the coefficient of variation in partial analyses is 5-7%.

We successfully tested the method developed in an investigation of the pharmacokinetics of I. Figure 4 presents as an example the results of a study of the dynamics of the concentrations of the drug in the blood serum and curves of the cumulative excretion of unchanged I with the urine in two patients after taking 40 mg I internally.

The proposed method can be used to analyze the relationship of the diuretic and saluretic effects to the concentration of I, to diagnose certain disorders of kidney function, to investigate the biological accessibility of new drug forms of I, and to optimize and individualize the dosage schemes of the drug.

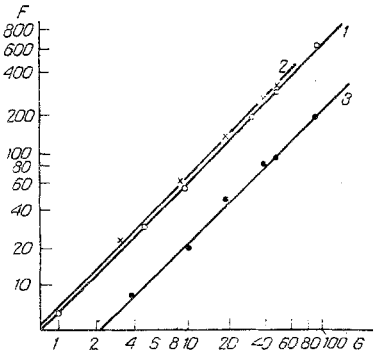

Fig. 3

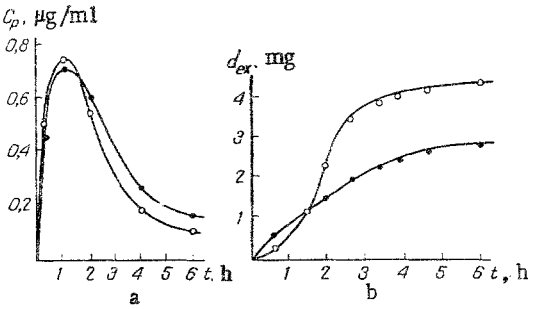

Fig. 4

Fig. 3. Graph of the relationship between the amount of I (G, in ng) in a spot on the TLC and the area bounded by the corresponding peak (F, in arbitrary units) on the densitogram obtained by scanning the plate in a fluoresence system (excitation at 279 nm, recording of transmission λ > 390 nm). Logarithmic coordinates.

Fig. 4. Dynamics of the concentrations of I in the blood (a, along y axis C_p) and cumulative curve of the excretion of I with the urine (b, along y axis d_{ex}) after taking 40 mg I internally (observations 1 and 2).

EXPERIMENTAL

Instruments. A KM-3 chromatogram-spectrophotometer (Opton, Federal Republic of Germany) was used fot TLC; measurement was performed in a fluorescence system. A sandwich chamber from Kodak was used for developing the TLC plates.

Reagents. Methanol for precipitation of proteins was cp grade. Silufol plates 15 × 15 cm, layer thickness 0.25 mm, without fluorescent indicator; before application of the samples, the plates were washed with methanol and dried in air for 20 min. After application of the samples, the TLC plates were developed in the system chloroform–methanol–glacial acetic acid (89:6:5); the components of this system were cp grade, and the distance traveled by the front 12-13 cm.

Standards. 4-Chloro-5-sulfanilanthranilic acid (III), one of the main metabolites, was produced by acid hydrolysis of I (heating of a 10% solution of I in concentrated sulfuric acid at 100°C for 3 h). The II was analytical grade. Standard solutions were prepared by dissolving 100 mg I, II, and III in 100 ml of ethanol, and they were diluted before use the requisite number of times with distilled water. The standard solutions are stable for a month.

Analytical Procedure. To 0.3-0.5 ml of blood serum (exact volume), a double volume of methanol is added; the precipitated proteins are centrifuged, and the supernatant liquid (50-100 μl, exact volume) or urine specimen (10-30 μl exact volume) is applied on a Silufol plate. The samples are applied in 5 μl portions with a microsyringe; after each application the spots on the plate are dried with a stream of air. Dilute standard solutions of I, II, and III are applied on the same plate for calibration. The plate is developed in the system indicated above. The following values were obtained for R_f: for I 0.58; for III 0.29; for II 0.83. The plate developed was dried in air for 15 min and then scanned on a KM-3 instrument. Conditions of scanning: the plate moves at a speed of 120 mm/min; mercury lamp, a monochromatic F39 filter used in the recording of the emission, lined with 8 mm.

The concentration of the preparation (in micrograms per ml) and its metabolite in the blood serum (C_p) and urine (C_m) are calculated according to the formulas:

$$C_p = \frac{b_1 \cdot F\,(V_p + V_c) \cdot 1000}{V_\Phi \cdot V_p}; \quad C_M = \frac{b_2 \cdot F \cdot 1000}{V_M},$$

where b_1 and b_2 are the transport coefficients of the area bounded by the peak (F) and the amount of the substance in the spot (in ng; determined according to a calibration curve); F is the area bounded by the peak on the densitogram; V_f or V_m is the volume of the protein-free filtrate of the blood serum or the volume of urine (in μl), respectively, applied on the blood serum or the volume of urine (in l), respectively, applied on the plate; V_p and

V_c are the volumes of blood serum and methanol, respectively, for the precipitation of protein (in ml).

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A MICROBIOLOGICAL METHOD OF DETERMINING PANTOTHENIC ACID IN READY-MADE DRUG AGENTS

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At the present time, for a quantitative determination of pantothenic acid (PAA) in various drug forms, microbiological methods use the lactic acid bacteria *Lactobacillus arabinosus*, *L. casei*, and *L. plantarum* [1, 2], as the test cultures. In our country PAA is determined by drug agents by a test-tube method with a test culture of *Saccharomycodes ludwigii* [1, 3, 4]. *S. carlsbergensis* and *S. cerevisiae* can also be used for these purposes [5, 6], but they are distinguished by lower specificity of the growth reaction to the vitamin, since they react appreciably to β-alanine and some of its peptide derivatives.

It is noted that the existing microbiological methods of quantitative determination of PAA are insufficiently specific and extremely laborious. Therefore, searches for a simpler and readily reproducible method of determination of PAA are essential.

Our investigations were conducted along several lines. An attempt has been made to utilize the test microorganism *S. ludwigii* in a dish variant of the microbiological method of determining PAA. However, clear readily reproducible zones of intensification of the growth of the test culture could not be obtained. The test-tube variant of the method with the test culture of *L. plantarum* also did not give satisfactory results. This same culture was used by Japanese researchers in the development of a dish method of intensified culturing using paper disks [7].

We have developed a dish method of quantitative determination of PAA in ready-made drug agents (RDA) with the test microorganism *L. plantarum* ATCC 8014, using the principle of the three-dose method of determining the activity of antibiotics, described in the British pharmacopoeia (1968 edition) [8].

EXPERIMENTAL

The liquid medium recommended by the US Pharmacopoeia (15th edition, 1955) for the test-tube method, with an addition of 1.5% agar [2, 9], was used as the basic medium, and agarized medium for lactobacilli as the maintenance medium [2]. The inoculating medium was prepared from the basic medium by adding 20 μg/liter calcium pantothenate (CPN) to it.

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