New Potentialities of HPLC in Pharmacopoeian Analysis

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Modern status of pharmacopoeian HPLC analysis and its main shortcomings are discussed. The philosophy "special analytical method for each substance" has to be revised, and as an alternative, a principle of creation of universal methods realized by means of HPLC analyzers is proposed. A prototype of such analyzer is described. The device was designed on the basis of a Milichrome A-02 gradient chromatograph with spectrophotometric detector and reverse phase column.

Key Words: *high pressure liquid chromatography; UV spectroscopy; reverse phase chromatography; gradient HPLC; pharmacopoeian analysis*

High performance liquid chromatography (HPLC) is a very important method of pharmacopoeian analysis of substances and drugs. It has been used for 30 years and remains one of the most labor- and time-consuming and expensive methods. The main reason for this is conservative approach to the use of HPLC fixed in the leading Pharmacopoeias of the world: every HPLC procedure is developed by the authors of the Pharmacopoeia articles in accordance with their potentialities and knowledge. As a result, a "unique" analysis requiring a special chromatographic column, mobile phases, and method of calibration is needed for each substance. Even a relatively easy analysis requires a kit of accessory materials, highly qualified specialists, and takes many hours. For example, HPLC analysis of ampicillin according to European Pharmacopoeia takes more than 15 hours [9]. This state of the art largely impedes effective drug quality control.

An alternative to traditional HPLC is an approach, which is actively used by some toxicologists [4,6-8,11-14] and pharmacologists [1-3] for the analysis of many substances, including pharmacopoeian preparations. This approach consists in the analysis of all compounds from a certain list (20-500 names) in the same chromatographic system (reverse phase column, gradient elution, UF detection) precalibrated for each substance. The peaks of the studied sample chromatogram are identified by comparing retention time and UV spectrum characteristics with the corresponding parameters of reference substances determined previously. These reference parameters serve as the database, and the chromatograph for the analysis making use of the database is an HPLC analyzer. Obviously, the use of HPLC facilitates the procedure and accelerates analysis.

However, despite the obvious advantages of the idea of HPLC analyzers, it is not yet widely used in practice for at least two reasons. First, all analyzers were designed on the basis of nonstandard liquid chromatographs, are unique, and cannot be reproduced. Their databases cannot be "transferred" even to chromatographs with similar characteristics, because this requires the formation of a new database. Second, there is a problem of column standardization. It is known that reverse phase adsorbents even very similar by their characteristics show different selective activity towards many substances. Only the use of the same adsorbent (better from the same lot) guarantees the reproducibility of the retention time. Attempts at

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introducing different systems of relative retention indexes for the endless variety of chemical structures are baseless.

This prompted us to search for approaches to these problems and a prototype of reproducible HPLC analyzer was developed. This prototype is based on a Milichrome A-02 chromatograph (EkoNova Firm) with a two-syringe gradient pump, UV spectrophotometric detector, autodoser, and column thermostat [5]. Unlike other chromatographs, Milichrome A-02 is manufactured by a technology, which guarantees stability of its main technological characteristics in each device.

Another important characteristic of this chromatograph is that it is intended for 0.2-ml columns only.

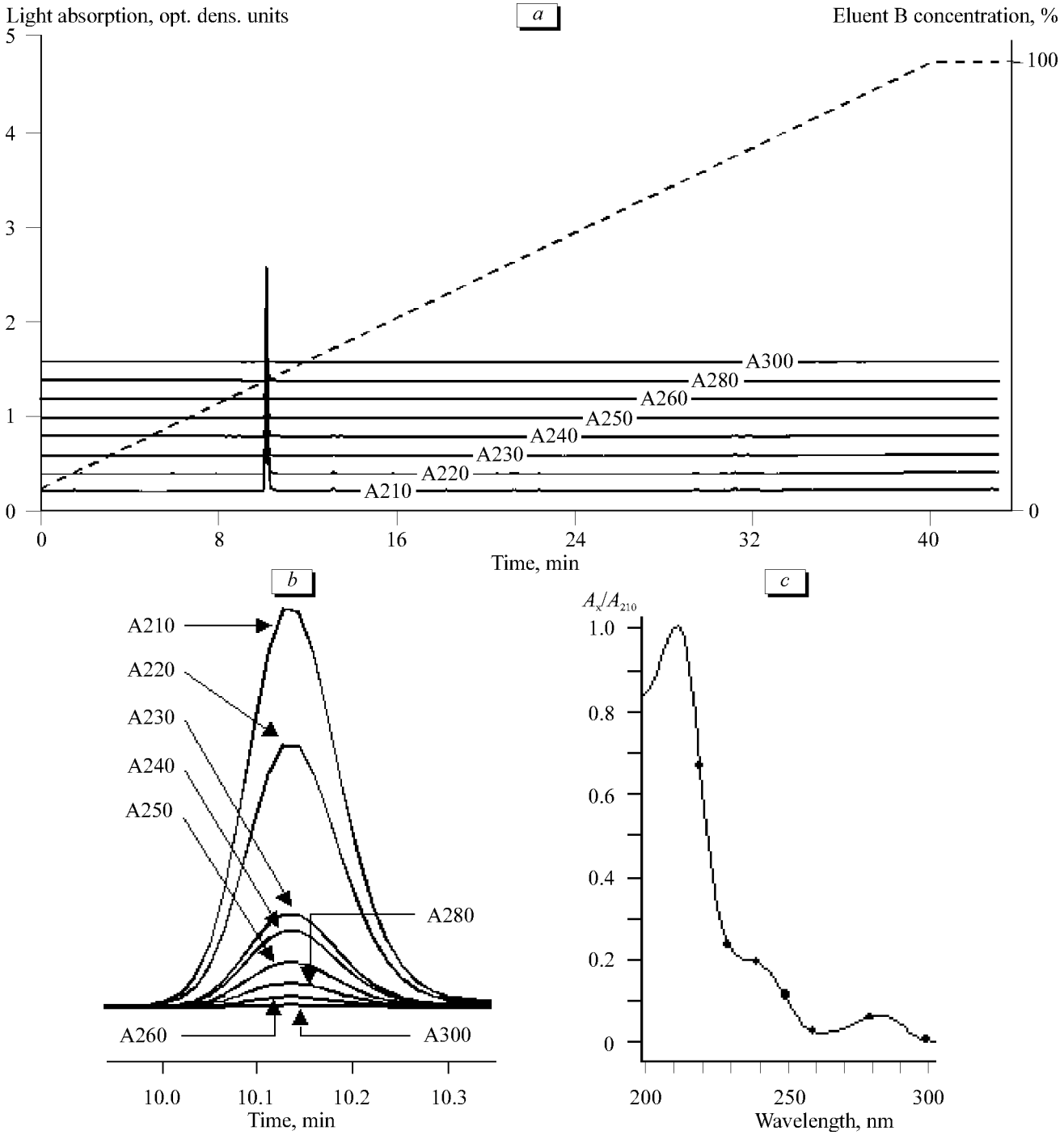

Fig. 1. A typical analysis performed on an HPLC analyzer. *a*) codeine chromatogram with detection at 8 wavelengths; *b*) magnified fragment of chromatogram; *c*) standardized UV spectrum of codeine recorded after flow arrest near the peak maximum. Dots show the $R=S_\lambda/S_{210}$ values on the spectrum found from the chromatogram.

This means that 5000 similar columns can be packed with just 1 kg of adsorbent of the same lot and used for several years without correcting the database. We used 2×75 mm columns packed with ProntoSIL-120-5-C18 AQ reverse phase (Bischoff Analysentechnik und Gerate GmbH). Their efficiency is at least 5000 theoretical plates, which ensures notable economy of expensive mobile phases in comparison with large columns. The adsorbent can be used in up to 1000 analyses without essential impairment of its characteristics, does not collapse in a mobile phase with high water content (up to 95%), and exhibits no ion-exchange properties towards amines due to residual silanol groups of silica gel.

Gradient elution is carried out with two eluents: eluent A (4 M $LiClO_4$-0.1 M $HClO_4$:H_2O, 5:95 ratio) and eluent B (acetonitrile for HPLC, Kriokhrom). These eluents are highly transparent in the short-wave UV band and contain no UV absorbing admixtures yielding extra peaks on chromatograms. The presence of acid in the mobile phase (pH 2.8) improves chromatography of carbonic acids, while high content of lithium ions improves amine chromatography.

UV detection is carried out simultaneously at 8 wavelengths: 210, 220, 230, 240, 250, 260, 280, and 300 nm. Eight peaks with the same retention time, but with different amplitudes directly proportional to the substance extinction correspond to each substance on chromatograms (Fig. 1). Seven standardized spectral parameters are calculated for each substance: the ratios of peak areas at wavelengths λ_2-λ_8 to the peak area at λ_1=210 nm ($R=S_\lambda/S_{210}$). These spectral R ratios together with the retained volume (V_R) are used for identification of the substance peak on chromatogram.

The conditions of HPLC are as follows: flow rate 100 μl/min, elution 40 min with linear gradient of 5-100% acetonitrile and then 3 min with 100% acetonitrile, column temperature 40°C, sample volume 4 μl, substance concentration in the sample about 0.2 mg/ml, solvents water, methanol, or their mixture.

The database was formed by chromatographic analysis of solutions of control substances of known purity (90-100%). An example of such a chromatogram (control substance codeine) is presented in Fig. 1. V_R, S_{210}, and R_1-R_7 values were calculated using Multichrome software (Ampersend Firm) attached to the chromatograph. S_{210} values were converted for 1 μg of the substance in the peak ($S_{210}^{1\ \mu g}$), and all data were recorded in a table in the order of V_R values increase; a fragment of this table is shown in the upper section

TABLE 1. Fragment of Database Table

Substance	V_R, μl	$S_{210}^{1\mu g}$	$R=S_\lambda/S_{210}$ at λ, nm						
			220	230	240	250	260	280	300
Barbital	933	40.46	0.62	0.09	0.03	0.01	0.01	0.00	0.00
Analgin	940	22.36	0.71	0.61	0.67	0.70	0.78	0.31	0.01
Phenylpropanolamine	956	53.56	0.14	0.01	0.01	0.03	0.03	0.01	0.00
Norpseudoephedrine	974	47.89	0.13	0.00	0.01	0.02	0.02	0.00	0.00
Propylhexidine	984	—	0.43	0.30	0.17	0.04	0.01	0.03	0.00
Sulfasol	993	45.53	0.56	0.30	0.38	0.65	0.86	1.04	0.89
Codeine	1013	44.49	0.67	0.23	0.20	0.11	0.03	0.06	0.01
β-Phenylethylamine	1038	59.34	0.09	0.01	0.01	0.02	0.02	0.00	0.00
Ephedrone	1064	50.46	0.15	0.44	1.07	1.53	1.08	0.18	0.09
Pseudoephedrine	1069	60.60	0.14	0.00	0.01	0.02	0.02	0.00	0.00
Ephedrine hydrochloride	1073	44.64	0.14	0.00	0.01	0.02	0.02	0.00	0.00
Hexamidine	1074	39.19	0.49	0.15	0.03	0.02	0.02	0.00	0.00
Control substances									
Bromide ion	148	25.79	0.09	0.00	0.00	0.00	0.00	0.00	0.00
Uridine	202	41.14	0.56	0.26	0.44	0.85	1.14	0.41	0.00
Caffeine	779	—	0.42	0.25	0.15	0.15	0.30	0.38	0.01
Proserine	1006	34.71	0.43	0.05	0.01	0.03	0.05	0.00	0.00
m-Nitroaniline	1179	65.35	1.31	1.50	1.40	1.21	0.90	0.55	0.28
o-Nitroaniline	1518	74.08	1.69	1.74	1.07	0.57	0.39	0.80	0.30
Triphthasine	2322	39.31	0.58	0.58	0.73	1.20	1.55	0.09	0.17

Note. Control substances are standard mixture components.

of Table 1. In order to have additional information about the substance, complete spectrum of its solution was recorded at 200-300 nm after the flow was stopped near the peak maximum (Fig. 1, *c*).

The peak of substance *X* is identified by means of this table as follows:

— candidate substances are selected in the ±0.05 V_R interval by V_R value;

— R ratios for substance *X* are compared with R values for candidate substances with consideration for the error (±0.02 for R=0.5-1.5 and ±0.01 for R=0.01-0.05);

— if all identification parameters coincided (*i.e.* the substance is identified), the content of the substance in the sample is calculated by the S_{210} and $S_{210}^{1\mu g}$ values.

The correctness of this method is regularly verified by chromatography of a special reference multicomponent solution (Fig. 2). By comparing the results of check-up with the data in Tables 1 and 2 (lower part), it is possible to make a conclusion about the status of all components of the chromatographic system. The components of the solution are chosen so that chromatographic and spectral parameters of each of them are in a certain way related to different parameters of the entire chromatographic system. Let us note some of these relations:

— bromide ion V_R characterizes free volume of the column. If it is 5% less than the value in the table, the system is not hermetic;

— a 0.02 deviation of $R=S_{250}/S_{280}$ value for uridine indicates disorders in the detector settings near λ=260 nm;

— a 0.05 increase of $R=S_{250}/S_{280}$ value for caffeine indicates that deviation of the linearity of the detector during measurements of absorption of solutions of more than 8-10 opt. dens. Units surpassed 2%;

— a 100 µl increase of V_R for proserine with simultaneous increase of the peak asymmetry indicates the appearance of silanol groups on the adsorbent surface as a result of its hydrolysis;

— a 0.05 deviation of $R=S_{250}/S_{280}$ value for *m*-nitroaniline indicates that the eluent pH value is wrong;

— deviations of any R values for *o*-nitroaniline higher than permissible indicates wrong settings of the detector in this or that spectrum. Deviation of this substance's V_R by more than 50 µl indicates improper formation of the gradient by the chromatograph pumps;

— increase of the peak asymmetry for triphthasine indicates that the mixer failed.

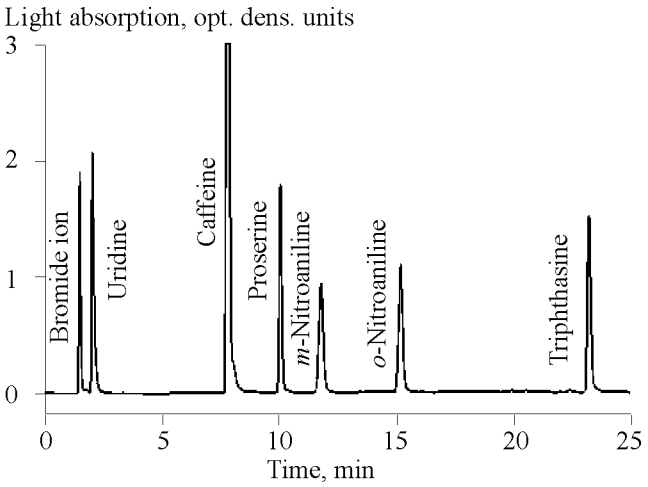

Fig. 2. Separation of control substances for validation of the analytical method. Chromatogram shows absorption of eluate only at λ=210 nm.

By the present time a prototype analyzer was tried at the Pharmacopoeian Committee of Ukraine (database for more than 150 substances), in pharmacokinetic studies [10], at Department of Toxicological Chemistry of I. M. Setchenov Moscow Medical Academy, and at Institute of Pharmacology, Tomsk Research Center, Siberian Division of Russian Academy of Medical Sciences.

Introduction of HPLC analyzers into practice of pharmacopoeian analysis is very important. It can be realized by adding a general pharmacopoeian article,

TABLE 2. Significance of Chromatographic Parameters for Components of Control Mixture

Substance	V_R, µl	$w_{h/2}$, µl	h_{210}, opt. dens. units	S_{210}, opt. dens. units×µl	$A_{5\%}$
Bromide ion	148	8.5	1.9	18.12	1.31
Uridine	202	10.4	2.1	27.66	2.14
Caffeine	779	10.1	18.3	209.23	1.18
Proserine	1006	11.3	1.8	22.85	1.16
m-Nitroaniline	1179	20.3	0.9	20.82	1.03
o-Nitroaniline	1518	19.9	1.1	23.93	1.05
Triphthasine	2322	15.0	1.5	25.88	1.27

Note. h_{210}: height of peak on chromatogram at λ=210 nm; $w_{h/2}$: width of peak at the level of 0.5 h_{210}; $A_{5\%}$: asymmetry of the peak at the level of 0.05 h_{210}.

describing the algorithm of database formation and algorithm of its utilization, into Pharmacopoeia of the Russian Federation. This method of HPLC analysis can serve as an alternative to currently used methods. Use of HPLC analyzers at laboratories of drug quality control is particularly important, as these laboratories have to analyze such a variety of substances, which can be hardly analyzed by means of traditional HPLC.

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