USE OF DUAL DETECTORS FOR THE DETERMINATION OF ALIPHATIC SULFHYDRYL COMPOUNDS BY HPLC

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Various aliphatic compounds containing mercaptyl groups are used in medicine as drug preparations: acetylcysteine, penicillamine, unithiol, cysteine, etc. [1].

High-performance liquid chromatography (HPLC) is a modern method which allows improved monitoring of drug preparations as well as compounds used for biopharmaceutical analysis. Various methods are known for the determination of acetylcysteine, penicillamine, and cysteine both in drug preparations and in biological fluids using HPLC. The detection methods used include spectrophotometric [5, 8], electrochemical (before [10-13] and after derivitization [6, 14]), fluorometric (after derivitization) [4, 7, 9], and polarographic [3] techniques.

In this work, we present the use of both spectrophotometric and electrochemical detection for the HPLC of thiols and disulfides using the Soviet-made "Millikhrom" chromatograph and the EMD-10 electrochemical microdetector. The use of dual detectors in the HPLC of drug-based thiols increases the selectivity of the analysis and provides additional information.

EXPERIMENTAL

Reagents. The following solvents were used: ethanol, redistilled; water, double distilled. Sodium dihydrogen phosphate ("pure" grade) was recrystallized from doubly distilled water. The phosphate buffer solution was a 0.01 M aqueous solution of sodium dihydrogen phosphate adjusted to pH 2 with orthophosphoric acid (85%; "specially purified" grade). As samples we used penicillamine, penicillamine in capsule form, penicillamine-disulfide (PO VNII antibiotikov), unithiol (PO "Oktyabr"), acetylcysteine ("Berlin Chemie"), cysteine hydrochloride ("Serva"), cystine ("Reanal"), dithiothreitol ("Merck"). Sample solutions at concentrations of 0.1-1 mg/ml were prepared directly by chromatography in the same mixture of solvents used for the mobile phase.

Materials and Chromatographic Conditions. We used the "Millikhrom" microcolumn liquid chromatograph. A steel column, 62 × 2 mm, was packed with Nucleosil C-18 sorbent having a 5-micron particle size ("Macherey-Nagel", Germany). Mobile phases were prepared by mixing the phosphate buffer solution and ethanol in various volume ratios. The ratios of these components selected for mobile phases for the chromatographic separations of various thiols and their homodisulfides are indicated in the text of the illustrations. Mobile phases were prepared immediately before use and were degassed using an aspirator. The compounds studied were chromatographed under isocratic conditions at a volumetric flowrate of 50 or 100 µl/min and about 20°C. Between 2 and 10 µl of sample was injected. Both spectrophotometric and voltammetric detection were used in this work. The spectrophotometric detector for the "Millikhrom" chromatograph was used at a wavelength of 210 nm, which is close to the transmission limit for the mobile phases used. At this short wavelength the spectrophotometer comes close to being a universal detector, which increases the amount of information that can be gathered by the detector. To carry out only voltammetric detection, the column was detached from the spectrophotometric cuvette and installed (as recommended in [2] directly into the inlet port of the EMD-10 detector, using a short length of teflon capillary as a seal. To carry out sequential, dual detection, the EMD-10 detector was joined to the outlet of the spectrophotometric cuvette by a very short piece of capillary.

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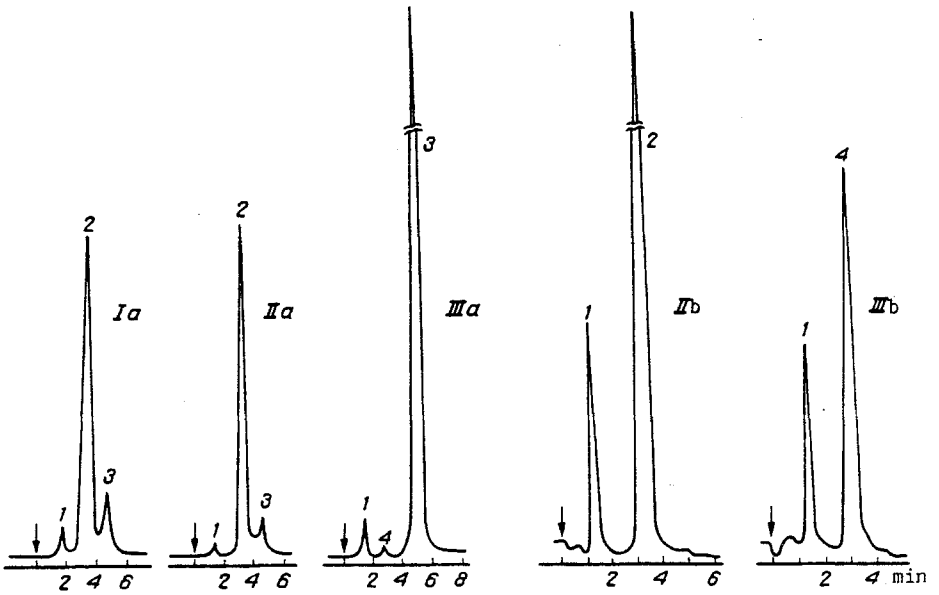

Fig. 1. Chromatograms of penicillamine and its disulfide. Mobile phase: phosphate buffer (pH 2.0). Eluant flowrate: 100 µl/min. Detection: spectrometric at 210 nm (a) and voltammetric at 0.8 V (b). 1, 4) Unidentified peaks; 2) penicillamine; 3) penicillamine disulfide.

In contrast to the commonly-used three-electrode voltammetric detectors, the EMD-10 detector (Laboratory Instruments, Prague) is a novel type of detector having two electrodes, i.e.; lacking a reference electrode. The platinum working electrode is polarized using a potentiostat. The polarization potential applied to the working electrode can be 0.8, 1, or 1.2 V. Since the working electrode is thus positively charged, we subsequently used oxidative polarization potentials. The voltammetric detector was set at the working electrode's lowest potential value, 0.8 V, for most of the experiments; in a few cases, it was set at 1 V.

Two automatic recorders were used to chart the chromatograms, i.e., one connected to each detector.

The use of an electrochemical detector with the "Millikhrom" chromatograph has been described earlier in the literature. The use of various interchangeable detectors is a known approach in chromatography, however, the advantages of microcolumn chromatography can be realized only through miniaturization of the system, especially the detector cell volumes [2]. The use of detectors having cell volumes of several microliters in conjunction with short microcolumns will result in significant spreading of the chromatographic zones. The EMD-10 microdetector used in our work has an analytical cell whose effective volume (4 nanoliters) is sufficiently low, and was joined to the short microcolumns of the "Millikhrom" chromatograph. As described in the experimental section, since the detector and column together had a minimal combined volume, significant distortion or spreading of the peaks was not observed.

Chromatographic analysis of penicillamine, acetylcysteine, unithiol, or cysteine preparations should provide information about the concentrations of both the drug substances themselves as well as those of the known impurities, most of which are oxidation products (the corresponding homodisulfides). The determination of drug compounds in blood plasma requires their separation from the usual plasma components, particularly the amino acids cysteine and cystine. We examined the chromatographic separation of the indicated preparations with dithiothreitol (1,4-dimercaptobutane-2,3-diol), which is used as an antioxidant for thiols during their determination in biological fluids.

The chromatographic separation was carried out on an octadecylsilane reversed-phase column, as has been recommended by most of the literature sources. Water–alcohol mobile phases were also used in most of the methods developed, however, there is no systematic study in the literature data of the effects of the ratio of mobile phase components on separation;

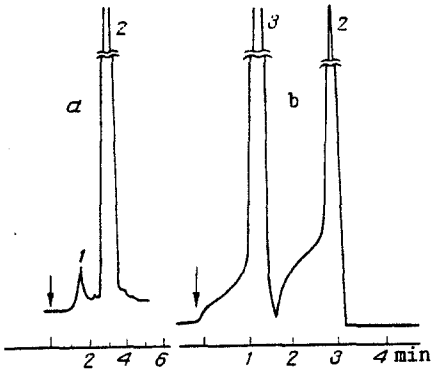

Fig. 2

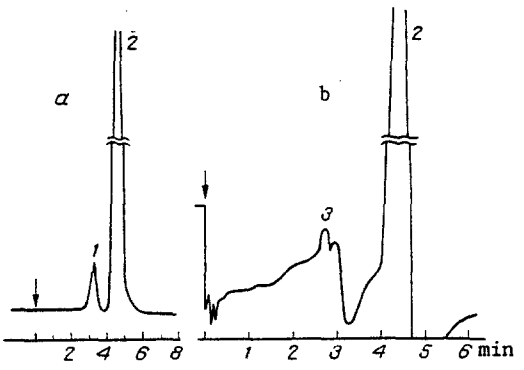

Fig. 3

Fig. 2. Chromatograms of unithiol. Mobile phase: phosphate buffer – ethanol (9:1). Eluant flowrate: 100 µl/min. Detection: spectrophotometric at 210 nm (a) and voltammetric at 0.8 V (b). 1, 3) Unidentified peaks; 2) unithiol.

Fig. 3. Chromatograms of acetylcysteine. Mobile phase: phosphate buffer (pH 2.0) – ethanol (8:2). Eluant flowrate: 50 µl/min. Detection: spectrophotometric at 210 nm (a) and voltammetric at 1 V (b). 1, 3) Unidentified peaks; 2) acetylcysteine.

only the final results are given. In this study, using phosphate–ethanol (1:9) as the eluant, all of the compounds studied (with the exception of dithiothreitol) were weakly retained on the nonpolar sorbent used. As the concentration of the organic modifier in the mobile phase is reduced, all compounds become more strongly retained, as would be expected in reversed-phase chromatography. The retention times and the differences between retention times for different compounds are increased simultaneously, which corresponds to an improvement in the separatory power of the chromatographic system. Mobile phases containing less than 50 volume percent ethanol are used in practice. Under these conditions the retentions increase in the order: cystine, cysteine (practically unretained) < pencillamine disulfide < penicillamine < acetylcysteine < unithiol < dithiothreitol, which is due both to increases in nonspecific interactions with the sorbent and reduction in solvation by the mobile phase. At very low or zero alcohol concentrations, the indicated retention order is changed somewhat, particularly in that penicillamine is retained more weakly than its disulfide.

Chromatograms are presented in Fig. 1 for samples of penicillamine (I), penicillamine in capsule form (II), and penicillamine disulfide (III). These chromatograms were obtained by sequential detection using spectrophotometric (a) and voltammetric at 0.8 V (b) detectors. Chromatograms I and II are practically identical. Minor peak 3 on these chromatograms was identified through the match of its retention time with that for penicillamine disulfide in chromatogram IIIa. This peak is not present in the chromatograms produced using the voltammetric detector, i.e., at the minimum potential of the platinum working electrode, 0.8 V, penicillamine is selectively detected in the presence of its disulfide.

The use of voltammetric detection at 0.8 V allows cysteine and cystine to be resolved in spite of their close retention times, since only the sulfhydryl compound cysteine gets oxidized. Other amino acids, alanine for example, also produce no peaks on the chromatograms using this detection mode.

The use of dual (spectrophotometric and voltammetric) detection produces two complimentary chromatograms, since each chromatogram may contain peaks which do not appear (do not register) on the chromatogram produced by the other detector. Comparison of these chromatograms may be useful for the identification and and determination of a large number of components, including impurities in the drug preparations. As an example, Fig. 2 shows chromatograms of unithiol produced using spectrophotometric (210 nm) and voltammetric (0.8 V) detection.

Voltammetric detection at working electrode potentials greater than 1 V is less selective for thiols and is accompanied by greater baseline drift than is the case at 0.8 V, however, this can also be useful for the identification and determination of all components (impurities) of drug preparations. As one can see in Fig. 3b, peak 3 registers on the chromato-

gram with voltammetric detection at 1 V, while it is absent from the chromatogram produced by the spectrophotometric detector. On the other hand, only the spectrophotometric detector (Fig. 3a) registers minor peak 1, which elutes after peak 3.

Thus, the use of voltammetric detection, as well as dual sequential spectrophotometric and voltammetric detection, for the chromatography of sulfhydryl drug compounds increases the selectivity and available information of the HPLC method, and also expands the potential uses of the "Millikhrom" chromatograph in pharmaceutical and biopharmaceutical analysis.

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RAPID DETERMINATION OF PANTHENOL BY HPLC

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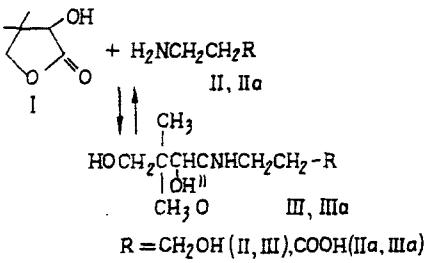
The synthesis of the B_3 group of vitamins is accomplished by condensation of pantolactone (I) with the corresponding component II [6]:



Indirect methods based on the determination of the hydrolysis products of III or IIIa are known [1] for analysis of the target compounds. In addition, a chromatographic method (GLC) is used for the determination of I [2, 4].

Recently, reports have appeared concerning the use of reversed-phase HPLC for the direct determination of pantothenic acid and its salts, without the need for preliminary hydrolysis [3, 7-9].

The goal of the present study was to develop a direct HPLC method for the determination of panthenol as a monitoring technique in the industrial production of vitamin B_3.

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