SEPARATION OF PANTOTHENIC ACID DERIVATIVES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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A variant of the use of reversed-phase high-performance liquid chromatography is described which permits the separation of pantothenic acid derivatives. The stationary phase used was a μBondapak-C_{18} (4.1 × 250 mm column; 4.6 × 50 mm precolumn). Elution was performed in the isocratic regime using as mobile phase 20 M potassium phosphate buffer (pH 5.0)–methanol (91.5:8.5). The rate of elution was 1 ml/min. Retention times in the column for phosphopantothenate, pantothenate, phosphopantetheine, CoA, and dephosphoCoA were about 3.5, 6, 10.5, 16, and 42 min, respectively. This method, with radioactive detection, can be used for the analysis of pantothenic acid derivatives in liver extracts. One hour after white rats had been injected with [^{14}C]pantothenic acid, the above-mentioned components (with the exception of dephosphoCoA) contained the label in a ratio of 4:18:54:24.

The development of pantothenate-containing preparations for medical and agricultural use in our country and abroad, the search for new methods of their production, and the increasing demands on their purity and stability have still not been backed up by an adequate methodological basis for their quantitative analysis [1]. The main problem in this respect is the absence of an effective method for separating derivatives of pantothenic acid (PAA), its possible metabolites, and its coenzyme form, which is of fundamental value in the standardization of preparations when they are obtained by chemical or biotechnological methods, and also in the investigation of biotransformation.

As is known, PAA metabolizes in the organism to several intermediate products with the formation of the final products of metabolism – CoA and phosphopantetheineproteins [1]. The first reaction of this multistage process brings about the phosphorylation of PAA with the formation of phosphopantothenate (PPA), which, interacting with cysteine, forms phosphopantothenoylcysteine. The decarboxylation of the latter forms phosphopantetheine (PPN). The interaction of PPN with ATP leads to the formation of dephospho-CoA. Then the dephospho-CoA is phosphorylated with the formation of CoA [1, 2].

Various methods for the quantitative determination of PAA derivatives are known – microbiological, enzymatic, thin-layer and gas-liquid chromatography, and gel filtration [1]. However, the above-mentioned methods are distinguished by low specificity, inadequate efficiency, and lengthiness of the analysis [1]. The most promising method of detecting PAA derivatives is high-performance liquid chromatography (HPLC). At the present time, several variants of the use of this method for separating and analyzing PAA-containing compounds have been described in the literature [4-8].

In the present paper we describe a method of separating PAA derivatives by reversed-phase HPLC. Brief information on this method has been reported previously [9, 10].

The results of the chromatography of a mixture of standard PAA derivatives are shown in Fig. 1a. As can be seen from the figure, the method of HPLC that we have used permitted the separation of PAA, PPA, PPN, dephospho-CoA, and CoA. These vitamin derivatives are eluted in the following sequence: PPA, PAA, PPN, CoA, and dephospho-CoA, with retention times of about 3.5, 6, 10.5, 16, and 42 min, respectively. Thus, this method has permitted the separation of PAA derivatives using isocratic elution, while gradient elution is currently used for this purpose [7, 8].

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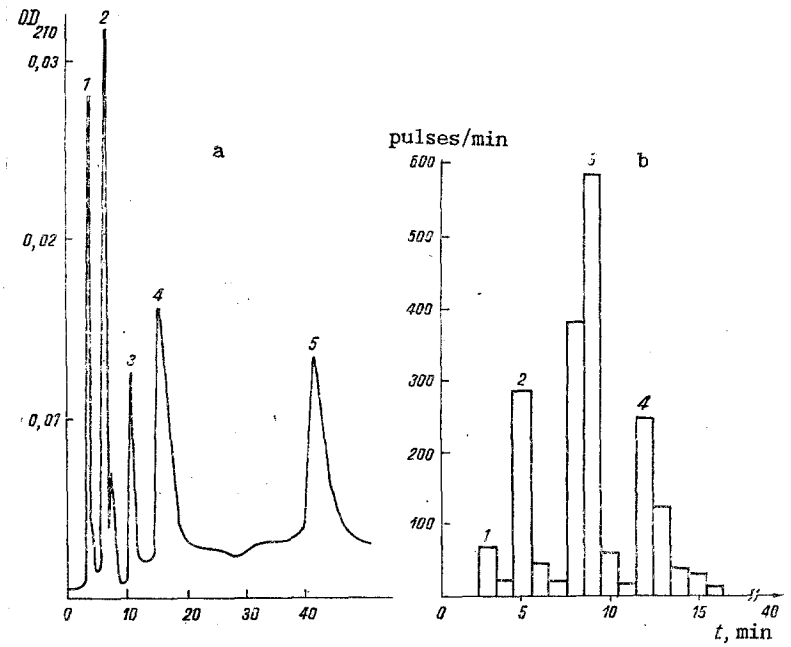

Fig. 1. HPLC of a mixture of standards of PAA derivatives (a) and of a deproteinated liver homogenate treated as described in the Experimental part (b). μBondapak-C_{18} column; rate of elution 1 mm/min; fractions collected every 75 sec; 1) PPA; 2) PAA; 3) PPN; 4) CoA.

In addition, we have studied the possibility of using the method of separating PAA derivatives under consideration for their analysis in a rat liver homogenate (i.e., for studying biotransformation). For this purpose, the experimental animals were first injected with [^{14}C]PAA in a dose ensuring the necessary level of activity in the organs, and after 1 h the animals were decapitated, after which a homogenate was obtained which was then worked up in several stages as described in the Experimental part. The results of the chromatography of this preparation are given in Fig. 1b. Chromatography gave four radioactivity peaks. Judging from the chromatographic characteristics of the PAA-containing components, the first peak corresponded to PPA, the second to PAA, the third to PPN, and the fourth to CoA. No peak corresponding to dephospho-CoA could be detected. Nor was a peak shown on the chromatogram that could be assigned to 4'-phosphopantothenoylcysteine. As can be seen from the chromatogram, the proportion of the label incorporated in the PPN was about 54% of the radioactivity, in the PAA 18%, in the PPA 4%, and in the CoA 24%, i.e., 1 h after its injection into the animal organism a considerable part of the label was detected in the PPN.

The detection of PAA derivatives after the chromatography of a deproteinated liver tissue homogenate is possible only by means of a radioactive label. The spectrophotometric quantitative determination of these substances is marred by impurities absorbing in the ultraviolet region.

In this work we did not determine the amount of phosphopantothenoylcysteine; however, its amount in tissues is insignificant, being less than 1% in the heart [8].

The method proposed in the present paper may prove effective in monitoring the purity of preparations, in determining PAA derivatives in pharmaceutical products and in biotechnological systems, and also in pharmacokinetic studies. This method permits the rapid (in less than 1 h) and fairly effective separation of PAA derivatives.

EXPERIMENTAL

The following preparations were used in the investigation: PAA from Koch-Light; [^{14}C]PAA from Du Pont-NEN; PPN Ca salt from Daiichi Seiytu. PPA synthesized in A. V. Lysenkova's laboratory; dithiothreitol (DTT) from Reanal, dephospho-CoA from P.-L. Biochemicals; and CoA from Ferak. The remaining preparations were of domestic production and of KhCh ["chemically pure"] or ChDa ["pure for analysis"] grade. The KH_2PO_4 and methanol for HPLC were subjected to additional purification.

Chromatography was performed in the isocratic elution regime in a Liquochrom 2010 chromatograph with a UV detector (Hungary) on a μBondapak-C_{18} (10 μM) column (4.1 × 250 mm) with a μBondapak-C_{18} precolumn (4.6 × 50 mm). The mobile phase was a 91.5:8.5 mixture of 20 mM potassium phosphate buffer, pH 5.0, and methanol. Detection was carried out with a UV detector at a wavelength of 210 nm or from the radioactivities of the fractions. The rate of elution was 1 ml/min. The buffer and the methanol were degassed with the aid of a "Millipore" filter system (USA). The buffer was filtered through a "Millipore" filter of the HATF 047 type (0.45 μm) and the methanol through a Durapore filter of the GVWP 4700 type (0.22 μm). To study the possibility of analyzing PAA metabolites in rat liver extracts, [^{14}C]PAA was injected intraperitoneally in a dose of 1.2 nmole/kg ($80 \cdot 10^6$ pulses$\cdot$min$^{-1}\cdot$kg^{-1}) 1 h before decapitation. After the cessation of blood circulation, the liver was rapidly extracted and frozen in liquid nitrogen, and then an aqueous homogenate (1:4) was prepared which was boiled in the water bath for 6 min. After cooling, the protein was eliminated by centrifugation on a K-24 centrifuge at 18,000 rpm for 10 min. DTT was added to the supernatant in a final concentration of 200 mM. The samples were incubated at 4°C for 1 h and were then filtered through a Sep-Pak C_{18} column that had first been washed with methanol and water. After filtration, the supernatant was collected and evaporated in a rotary evaporator to one-fifth of its initial volume, and 20 μl of this solution was injected into the chromatograph. Fractions were collected every 75 sec. The rate of elution was 1 ml/min.

CONCLUSIONS

PAA and its metabolic derivatives – PPA, PPN, CoA, and dephospho-CoA – have been separated by reversed-phase HPLC using spectrophotometric detection and detection based on radioactivity in an isocratic elution regime. The metabolic biotransformants of [^{14}C]PAA in a rat liver extract have been analyzed with radioactivity detection.

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