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USE OF HPLC FOR INCREASING THE MOLAR SPECIFIC ACTIVITIES OF TRITIUM-LABELLED NUCLEOSIDES

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SUMMARY

The possibility of chromatographic separation of different isotopic forms of tritiated nucleosides has been demonstrated. Using a reversed-phase column in 3-5% solutions of ethanol in water the labelled nucleosides eluted ahead of corresponding unlabelled analogs. Such isotope effects allow simultaneous fine purification of nucleosides and its fractionation according to molar specific activity value.

Key words: tritium-labelled nucleosides, chromatographic separation, isotope effects.

There are many articles in the literature which show a possibility of complete or partial separation of isotopically labelled molecules from corresponding non-labelled analogs on chromatographic columns (see, for example [1-3]). It seems appropriate to use the isotope effects for obtaining preparations with high molar specific activities on the stage of final chromatographic purification.

The object of the present work was an investigation of using HPLC for fractionation of tritium-labelled nucleosides according to its molar specific activity values.

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EXPERIMENTAL

Chromatographic separations were carried out on a column (250x4,0 mm) with Lichrosorb RP18 (5 μm) and appropriate guard column. Efficiency of the column was near 14,000 theoretical plates. A LKB liquid chromatograph was used for an eluent supply and UV-detection (254 nm). Eluents: 0.05M buffer solution of triethylammonium bicarbonate (pH=6.0) with 2% of acetonitrile or 3-5% solutions of ethanol in water. Tritium-labelled ribo- and deoxyribonucleosides, adenosine (Ado), uridine (Urd), guanosine (Guo), thymidine (Thd) and inosine (Ino) were prepared in our laboratory by following methods: solid-phase catalytic exchange with gaseous tritium [4,5] (compounds 1-3, 6, 7 in Table 1); catalytic dehalogenation of Br-precursor (compound 4 in Table 1); enzymatic [6] (compounds 5 and 8 in Table 1); liquid-phase catalytic reduction of carresponding 5-cyano-derivatives [7] (compounds 9 and 10 in Table 1). The labels locations were determined by ^{3}H-NMR spectroscopy (Bruker AC250, 266.8 MHz). In the text below the positions of molecules are indicated in which not less than 10% of hydrogen atoms are substituted by tritium.

Initial separation of the reaction mixtures were performed by a low pressure column chromatography on Dowex 1x8.

Radioactivity of fractions, obtained after chromatographic separations was measured by liquid scintillation counting using the internal standard method. Chemical homogeneity and identity to unlabelled substance were tested by the methods of UV-spectrometry and thin-layer radiochromatography. The relative error of specific activity measurement was less than 6% in all cases.

RESULTS AND DISCUSSION

On the chromatographic curves of tritium-labelled nucleosides we did not observe any obvious indications of the separation of

different isotopic forms. Nevertheless, following analysis of fractions, collected during elution of the nucleoside peak shows that a separation of the molecules according to their molar specific activity takes place. Outgoing peak of nucleoside was divided onto two fractions - from the beginning to the maximum and from the maximum to the end of the peak and the relative molar specific activities of the nucleoside in the two fractions was used as characteristic of enrichment. The results of experiments are shown in Table 1. In all cases we observed an enrichment of the head fractions of peak by the compound with higher molar specific activity. In the column "Output" of Table 1 a part of the total peak activity is shown which falls within given fraction.

In most cases for high performance separation the products were taken which had been obtained after tritium labelling and initial purification by a method of usual column chromatography. In such modification a simultaneous fine purification of the product and its fractionation according to molar specific activities are possible. The separated fractions had a radiochemical purity of not less than 96%.

Choice of a mobile phase composition was determined by necessity of its complete removal after fractionation on column, and from this point of view dilute ethanol solutions in water have an essential advantage over mobile phases containing volatile buffer systems. We have not observed any reduction in the efficiency of purification when buffer solution of triethylammonium bicarbonate was replaced by ethanol solution. Increase of ethanol concentration above 5% leads to very narrow peaks, which are fractionated with difficulty. A decrease of ethanol content below 3% allows wider peaks to be obtained, but accompanied by noticeable loss of nucleosides on column. Hence, the optimum ethanol concentration for purine nucleosides is 5% and that for pyrimidine nucleosides is 3%.

If required it is possible to obtain narrower fractions of labelled compound with corresponding decrease of the compound

TABLE 1.

Isotope effects in [^{3}H]nucleosides chromatography

RUN No.	COMPOUND	ELUENT[a]	OUTPUT,%[b] fract.1	fract.2	SPECIFIC ACT., Ci/mmol initial[c]	fract.1	fract.2	ENRICHMENT FACTOR[d]
1	[4′,5′,2,8-^{3}H]Ado	A	70	30	65.3	71.0	55.0	1.29
2	[4′,5′,2,8-^{3}H]Ado	A	40	60	76.4	92.2	68.6	1.34
3	[5′,2,8-^{3}H]Ino	B	49	51	62.5	72.6	55.2	1.32
4	2′-d[8-^{3}H]Guo	C	29	71	22.8	24.2	22.4	1.08
5	2′-d[2′,8-^{3}H]Guo	C	76	24	34.3	37.7	26.8	1.41
6	[5,5′,4′-^{3}H]Urd	B	51	49	58.4	61.8	55.2	1.12
7	[5,5′-^{3}H]Urd	B	38	62	29.2	32.1	27.7	1.16
8	[2′-^{3}H]Thd	B	49	51	22.4	24.4	20.7	1.18
9	[*methyl*-^{3}H]Thd	B	39	61	59.8	66.7	56.1	1.19
10	[*methyl*-^{3}H]Thd	B	60	40	35.4	45.2	26.7	1.69

[a]Eluents: A - 0.05M triethylammonium bicarbonate (pH=6.0) with 2% of acetonitrile; B - 3% ethanol solution in water; C - 5% ethanol solution in water.

[b]A part of the total peak activity in the fraction.

[c]Calculated from the specific activity values of the fractions obtained.

[d]Calculated as a ratio of specific activity in the first fraction to that in the second fraction.

quantity in the each fraction. Thus, in a separation of [5′,2,8-^{3}H]inosine peak in four equal volume fractions the following results were obtained: the first fraction contained 10% of total peak activity and the molar specific activity was 102 Ci/mmol; the second fraction - 39% and 67.2 Ci/mmol, respectively;

the third fraction - 46% and 55.6 Ci/mmol and the forth fraction - 5% and 49.8 Ci/mmol. Extremely high molar activity of the labelled inosine in the first fraction is due to tritium exchanging with two equivalent hydrogen atoms in the position 5′ in the conditions of solid-state catalytic exchange reaction. Similar experiment with the separation of [5,5′,4′-^{3}H]uridine peak in three equal volume fractions gave: the first fraction - 8% of total peak activity and specific activity was 60.0 Ci/mmol; the second fraction - 80% and 56.8 Ci/mmol; the third fraction - 12% and 32.7 Ci/mmol, respectively. The radiochemical purity in all the fractions was not less than 96%.

The enrichment factor value shown in the last column of Table 1 does not present an absolute degree of isotopic separation. This value depends on homogeneity of initial mixture of labelled and unlabelled nucleoside forms and tends to unity with increase of the mixture homogeneity. This is shown by the runs 9 and 10, in which the same preparation of labelled thymidine was used but in the run 10 it had been diluted by unlabelled thymidine nearly two fold.

Nevertheless, taking into account initial molar specific activity of labelled compound the enrichment factor value gives information both on isotopic homogeneity of the preparation and on the possibility of chromatographic fractionation of the labelled molecules.

Our results show that in most cases the greatest separation of isotopic forms under the experimental conditions occure with purine nucleosides.

The observed differences in retention of labelled and unlabelled molecules are in agreement with a concept that a labelled compound appears more polar than corresponding unlabelled one [4] and is retained by reversed phase to a lesser extent.

This effect can be used for isolation of tritium-labelled nucleoside fractions with high molar specific activity.

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