ESTIMATION OF PYRIMIDINE DEOXYRIBONUCLEOSIDES IN URINE

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An original analytical method for estimating pyrimidine deoxyribonucleosides in human urine was developed. It combines column and paper chromatography together with a quantitative microbiological assay using *Lactobacillus acidophilus* R 26. The reproducibility of the method is greater than 90% for all pyrimidine deoxyribonucleosides, the lower limit of sensitivity is 2 μg/1 000 ml urine. The method is applicable to experimental and clinical analysis of urine.

This laboratory has studied for several years the possibility of determining the degree of radiation damage to an organism on the basis of the amount of pyrimidine deoxyribonucleosides excreted with the urine. Already some time ago a quantitative relationship between deoxycytidinuria and the radiation dose[1-4] was described and an explanation of its origin was presented[5,6]. The finding was confirmed fully by a number of authors[7-16] and the dependence of excretion of other pyrimidine deoxyribonucleosides with urine on the radiation dose was examined[17-22].

In the present paper we intend to describe a sensitive and reliable method for determining simultaneously deoxycytidine, thymidine and deoxyuridine in human urine.

EXPERIMENTAL

Material. Ion-exchange chromatography was done on Dowex 1-X 8 (200—400 mesh) from Serva and on Dowex 50-WX 8 (200—400 mesh) from Fluka. Paper chromatography was done on Whatman No 3. As standards for paper chromatography and for recovery experiments the following compounds were used: deoxycytidine hydrochloride (Koch and Light, England), thymidine (British Drughouses) and deoxyuridine (Calbiochem, USA). The chromatographic zones were detected under a UV lamp (Mineralight Model L).

Method. The pyrimidine deoxyribonucleosides were determined as shown in Scheme 1. Columns of Dowex 1 and Dowex 50 (2 × 10 cm) are prepared, washed each with 200 ml 0·1M formic acid. From the total amount of 24 h urine a 100 ml portion is taken and made acid with formic acid (98—100%) to pH 2·3. The urine is then passed first through a column of Dowex 1 and then through Dowex 50.

Preliminary experiments showed the total deoxycytidine to be trapped on Dowex 50 from which it is eluted with 500 ml 0·1M ammonium formate, made to pH 8·0 with ammonia. The eluate is

evaporated to dryness by distillation at reduced pressure at 35°C and, after dissolving in a minimum volume of distilled water it is placed on 4 sheets of Whatman No 3 (23 × 57 cm) together with standards of deoxycytidine and thymidine. The separation takes place in a mixture of n-butanol with water (86 : 14) in an atmosphere of ammonia (added to the bottom of the chromatographic jar) for 17—18 h, leaving the solvent to run over the sheet end. Zones with R_F values corresponding to the deoxycytidine standard are cut out and eluted with water. After evaporation to dryness the residue is dissolved in 50 ml distilled water which is the starting dilution for a quantitative microbiological assay.

Thymidine is partly trapped on Dowex 50 and partly appears in the eluate. Thymidine trapped on Dowex 50 is determined in a way similar to that for deoxycytidine. After paper chromatography, zones with R_F values of thymidine are cut out and eluted. The eluate residue is diluted for the quantitative determination proper with distilled water to 25 ml. The remaining part of thymidine in the eluate is determined as follows: The eluate is evaporated to dryness and the residue is dissolved and applied to 4 sheets or Whatman No 3 together with standards of tymidine and deoxyuridine. Paper chromatography, detection, elution of zones and concentration of the eluate are done as described above. The residue is dissolved in 25 ml water and combined with the thymidine fraction obtained before.

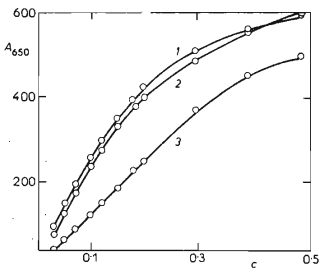


FIG. 1

Calibration Curves of Nephelometric Measurement of Increased Growth of *L. acidophilus* R 26 in Dependence on the Concentration of Pyrimidine Deoxyribonucleosides

C Concentration of compounds in μg/ml; A_{650} absorbance at 650 nm; *1* deoxycytidine; *2* thymidine; *3* deoxyuridine.

TABLE I

Results of Recovery Experiments in Human Urine with Known Amounts of Added Deoxyribonucleosides

Each value includes the mean error obtained from ten determinations.

Added μg/100 ml urine	0·5	1·0	2·0	3·0
Deoxycytidine	111 ± 2·9	112 ± 1·7	91 ± 2·8	81 ± 7·6
Deoxyuridine	—	—	—	—
Thymidine	—	—	—	—

After chromatography of the urine, the total deoxyuridine is in the eluate. Chromatographic zones corresponding to the deoxyuridine standard are cut out, eluted and concentrated. The residue of the eluate is dissolved for the determination proper in 50 ml distilled water.

Microbiological quantitative determination of the individual pyrimidine deoxyribonucleosides is done with the aid of *L. acidophilus* R 25 as described[6]. From the original volume of 50 ml, 0·25 ml samples are withdrawn and mixed with 0·25 ml cultivation medium[6]. After 17 h of incubation the growth of the microorganism is determined nephelometrically on a Beckmann DU spectrophotometer at 650 nm. Quantitative evaluation is done with the aid of calibration curves of the individual deoxyribonucleosides which are prepared always together with every determination (Fig. 1). To check the sensitivity of the method we used deoxycytidine[2-^{14}C] from Amersham, England, of a specific activity of 44 mCi/mmol. The resulting activity was determined by means of liquid scintillation in a Mark I scintillation spectrometer in the SLD 31 scintillator.

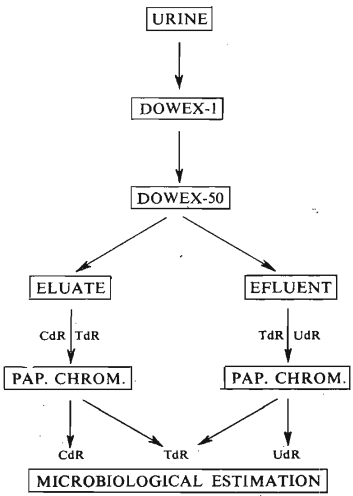
RESULTS AND DISCUSSION

The method was applied to the determination of pyrimidine deoxyribonucleosides added to 100 ml urine where the recovery was compared with samples of the same urine without standards added (Table I). It follows from the table that at higher concentrations of deoxyribonucleosides in the urine the recovery values generally decreased which may be due to nonlinearity of the calibration curve of microbial growth. In this case one must use greater dilutions of the starting sample for the microbiological assay. A role may be played by the capacity of the Dowex column which changes in dependence on the amount and quality of the individual components of the urine. To check the accuracy of the method and to achieve complete separation of the compounds we used deoxycytidine and deoxyuridine labelled at carbon 2 with ^{14}C and added them to the urine. After completion of the analysis the recovery was estimated from the final activity. Average recovery values found repeatedly were 90–92·5%. The present method proceeds from an earlier work[22] on the determination of deoxycytidine in human urine. The method was modified

TABLE I
(*Continued*)

5·0	10·0	20·0	30·0	Added µg/100 ml uridine
93 ± 5·1	74 ± 3·2	—	—	Deoxycytidine
95 ± 14·1	68·5 ± 4·7	48 ± 2·1	31·7 ± 4·7	Deoxyuridine
100 ± 15	71·9 ± 0·3	44 ± 0·3	36·8 ± 4·1	Thymidine

to make it possible to determine simultaneously the other two pyrimidine deoxyribonucleosides. Problems of a related character were taken up by other authors.



SCHEME 1

Method of Pyrimidine Deoxyribonucleoside Estimation in Human Urine
Abbreviations: CdR deoxycytidine, UdR deoxyuridine, TdR thymidine.

The method of I Wen Chan[10] based on a similar principle makes it possible to determine deoxycytidine alone. The method of Gerber and coworkers[16] employs repeated chromatography on ion exchangers and desalting of samples by extraction with methanol. The method achieved, however, substantially lower values of excreted deoxyuridine and thymidine. The present method is considered superior because it requires less time for routine determinations. Its reproducibility, lying between 90 and 92%, and its high sensitivity (2 μg/1000 ml urine) are taken as satisfactory for experimental and clinical analysis of urine. It appears to be suited for mammalian organisms with low values of deoxypyridine catabolites in the urine. Where the excretion is greater (like in the rat) it is preferable to use the conventional colorimetric or spectrophotometric procedures.

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