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ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR STUDYING THE METABOLISM OF BLOOD PLASMA PYRIMIDINE NUCLEOSIDES AND BASES: CONCENTRATION AND RADIOACTIVITY MEASUREMENTS

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SUMMARY

A rapid and efficient isocratic high-performance liquid chromatographic method for studying the metabolism of blood plasma cytosine, uracil, thymine, cytidine, deoxycytidine and uridine has been elaborated. For each compound this method can measure concentrations in the range 0.5–200 μM and determine radioactivity. All the pyrimidine compounds can be eluted in less than 18 min, and the total time elapsed between collection of the blood and completion of the analysis need not exceed 3 h. All measurements can be performed on 0.25-ml blood samples. Blood plasma pyrimidine concentrations were determined for the rat, the rabbit, the guinea pig, the dog and the healthy human. This method could be well applied to experimentation on small animals using radiolabelled pyrimidine derivatives, in order to study the metabolic pathways of nucleotides and nucleic acids. It could also be used to characterize certain illnesses or cases of toxicity created by a chemotherapy affecting the plasma level of pyrimidine bases or nucleosides.

INTRODUCTION

In mammalian cells, preformed pyrimidine bases and nucleosides can be phosphorylated via the "salvage" pathways to ensure the renewal of the pyrimidine nucleotide pools [1]. The identification of significant amounts of pyrimidine nucleosides (uridine and cytidine) in the blood plasma of several species, by high-performance liquid chromatographic (HPLC) analysis [2–5], has led to investigation of the link between the presence of such plasma precursors and the synthesis of nucleotides and RNA in tissues. At present this biosynthesis is a field of intensive investigation in cancer chemotherapy and liver disease. Nevertheless, the metabolism of the pyrimidine compounds of blood plasma is still not fully understood, since research has mainly been devoted to the nucleosides and little

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has been done on the bases [6,7]. Furthermore, the results of quantification of the amounts of these circulating compounds vary from one author to another, with values in rats, for example, ranging from 3.3 to 11 μM for cytidine concentration and from 1 to 32 μM for uridine [2,5,8,9]. The reasons for these discrepancies are unclear but may partly lie in differences in the treatment of the tissues together with incomplete HPLC resolution of peaks. Indeed, most HPLC procedures produce good peak resolutions for standard mixtures [5,10–14], whereas a high resolution of the peaks in the analysis of biological fluids is rendered difficult by the presence of a large number of unknown compounds.

Because of the absence of a simple and efficient procedure suitable for the analysis of all pyrimidine bases and nucleosides in blood plasma, we sought to elaborate an HPLC method capable of achieving within 20 min an accurate evaluation of the concentration of the following compounds: cytosine (Cyt), uracil (Ura), thymine (Thy), cytidine (Cyd), deoxycytidine (dCyd) and uridine (Urd). A rigorous preparation of biological samples used for the analysis of bases and nucleosides is also of great importance in view of the high sensitivity of the HPLC technique, which renders possible the specific activity determination for each of these compounds of the blood plasma from chromatograms by separate collection of eluted fractions without contamination or degradation. Using this procedure, the total time elapsed between collection of the blood and completion of the analysis, does not exceed 3 h.

We give the blood plasma concentrations of the main pyrimidine bases and nucleosides for rat, rabbit, guinea pig, dog and healthy human. This method can be adapted to in vivo experiments, using radiolabelled pyrimidine derivatives, for which good peak resolution is necessary in order to collect radiochemically pure compounds for specific radioactivity evaluation.

EXPERIMENTAL

Chemicals and labelled compounds

Bases and nucleosides were purchased from Sigma (St. Louis, MO, U.S.A.), heparin from Choay (Paris, France), hirudine from Diagnostica Stago (Asnieres, France) and pentobarbital from Clin-Midy (St.-Jean de la Ruelle, France). [U-^{14}C]Urd (393 mCi/mmol), [U-^{14}C]Cyt (300 mCi/mmol) [U-^{14}C]Ura (200 mCi/mmol) and [2-^{14}C]Cyt (54.6 mCi/mmol) were purchased from C.E.A. (Saclay, France). All other reagents for tissue treatment and chromatographic procedures were purchased from Merck (Darmstadt, F.R.G.).

Apparatus and HPLC conditions

The isocratic HPLC system consisted of a Model 380 Altex pump, an SPA-2A variable-wavelength UV detector (Shimadzu) with an 8-μl cell and a Model 71-25 syringe-loading injector (Rheodyne) with a 100-μl fixed-volume sample loop. Samples were injected by a syringe (S.G.E.) with a blunt 28-gauge needle.

Chromatography was carried out on an HPLC cartridge column (250 mm$\times$4 mm I.D.) prepacked with LiChrospher 100 CH-18/2, 5 μm particle size, purchased from Merck (F.R.G.). Peak areas were integrated and recorded using an SP-4100 (Spectra-Physics) printer plotted.

The isocratic system used an aqueous 0.01 *M* potassium dihydrogenphosphate solution prepared from ultrapure water, adjusted to pH 2.5 or 7.0 with pyrophosphoric acid or potassium hydroxide, filtered through a 22-μm filter (Millipore) and degassed using an ultrasonic bath.

The flow-rate was 1.0 ml/min in all experiments, and the working temperature was room temperature (20 ± 2°C). Absorbance was measured at 260 or 280 nm, and the sensitivity of the UV detection was 0.02 a.u.f.s. The quantitative determination of the compounds was carried out by external calibration.

Column equilibration and re-equilibration

Prior to use, the column was flushed for at least 20 min with 100% methanol, followed by a thorough flushing with ultrapure water (30 min), after which the column was allowed to equilibrate using a potassium phosphate buffer. After six plasma sample analyses, the column was flushed (30 min) with a mixture of methanol–potassium phosphate buffer (25:75) to wash out any remaining organic compounds. Finally, after sample analyses, the column was flushed with 25% methanol in potassium phosphate buffer, followed by ultrapure water and finally 100% methanol to inhibit bacterial growth.

Standard preparation

Standard solutions of Ura, Cyt, Urd, Cyd, dCyd, hypoxanthine (Hyp) and uric acid (UA) were prepared by dissolving high-quality pure standards in ultrapure water to approximate concentration. The exact concentration was then determined using a double-beam spectrophotometer (Beckman Acta III). Stock standard solutions of each compound were kept at −20°C when not in use. Calibration standard mixtures were prepared by the appropriate dilution of the stock solution in potassium phosphate buffer.

Animals and blood collection

Female Wistar rats, weighing 230–250 g, and female Dunkin Hartley guinea pigs, weighing 350 g, were kept under day–night cycle and had free access to standard laboratory food and drinking water. Blood sampling was carried out by means of a polyethylene catheter placed in right carotid artery of anaesthetized and heparinized animals. All sampling was started between 9 a.m. and 10 a.m. Blood samples were collected and immediately centrifuged.

In rabbits, blood samples were drawn by heparinized syringe from ear artery. In dogs, blood samples were drawn from awake animals by means of a catheter placed in the saphenous vein some days before.

In humans, blood samples were drawn by heparinized syringe from six laboratory members and sixteen other persons aged from 26 to 40 years.

Plasma sample preparation

Blood plasma was treated with a one quarter volume of ice-cold 6 *M* perchloric acid and kept at 4°C for 15 min. The precipitated proteins were removed by centrifugation at 13 500 *g*, and the supernatant was neutralized to pH 7.0 with 5 *M* potassium hydroxide. Potassium perchlorate was removed by centrifugation. De-

proteinized and neutralized plasma extracts can be kept frozen for several weeks at −20°C.

Labelling experiments

Labelled tracer doses of Ura, Urd, Cyt or Cyd dissolved in sterile 9 g/l sodium chloride were injected in 250-μl doses into the saphenous vein of rats. Then, 3 min before the end of experiments (60 min), the rats were given 500 I.U. (0.1 ml) of heparin intravenously, and the blood was collected from the carotid artery and treated as described above. For the in vitro experiments, both blood and blood plasma samples of rats were taken under sterile conditions from untreated animals, and hirudine or heparin was added to prevent coagulation. For each case, control samples, labelled Urd samples, and labelled Cyd samples were incubated for 30 and 60 min at 37°C, under sterile conditions, after which HPLC plasma sample preparation was carried out.

Labelling measurements

Six eluted fractions, corresponding, respectively, to (a) an under 3-min elution, followed by the peaks of (b) Cyt, (c) Ura, (d) Cyd, (e) dCyd, and (f) Urd, were collected drop by drop and their radioactivity measured by liquid scintillation spectrometry (A.C.S. Amersham). Each eluted fraction was accurately recovered, taking into account that in such conditions a volume of 60 μl was enclosed between the UV cell detector and the exit and that each drop contained 20 μl.

RESULTS

Chromatographic characteristics

The isocratic HPLC separation of Cyt, Ura, Cyd, UA, Hyp, dCyd, Urd and Thy, using a 100 CH-18/2 column and a 0.01 *M* potassium dihydrogenphosphate solution adjusted to pH 2.5 (1.0 ml/min flow-rate), was achieved in less than 18 min, as illustrated in Fig. 1. These conditions also give an optimal separation of the pyrimidine derivatives, and no interfering peaks were observed when the standard compounds were tested together. The chromatographic profile of a blood plasma sample of rat shows that Urd [retention time (t_R) = 12.8 min], dCyd (t_R = 10.5 min) and Cyd (t_R = 7.3 min) were equally separated with no interfering peaks. In contrast, the peaks of Cyt (t_R = 3.1 min) and Ura (t_R = 5.4 min) were incompletely resolved and their retention times were identical with those of several other unknown compounds. However, in certain cases (the guinea pig for example), the peak resolution was improved under the same conditions, except that the pH of the mobile phase was adjusted to pH 7.0 (Fig. 1).

Effect of mobile phase pH variation on the retention time

To study the effect of pH changes on the retention time, the molarity of the eluent was maintained constant at 0.01 *M*. Fig. 2 presents a plot of the adjusted retention time for seven compounds, collectively eluted, for values of the mobile phase pH ranging from 2.5 to 7.0. As the pH increased, the retention time for UA decreased greatly whereas for dCyd it increased significantly. Retention times for

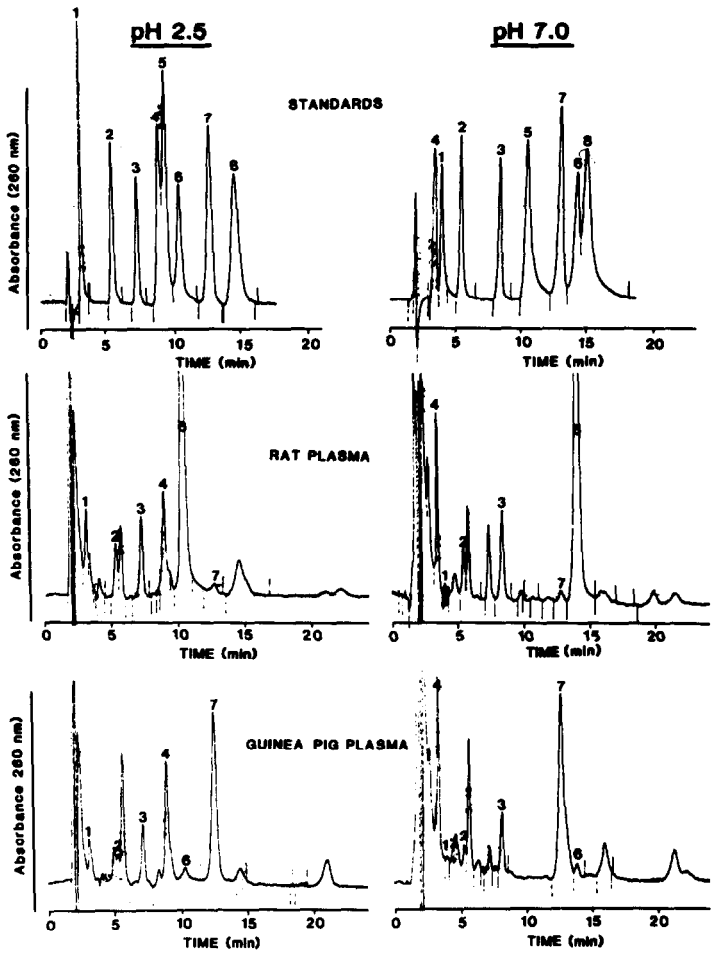

Fig. 1. Isocratic HPLC separation of bases and nucleosides from standard mixture or blood plasma samples. The concentrations of standards were in the range 5–15 μM. The dilution of plasma during the extraction procedure was 1.35. Injection volume, 100 μl; column, Merck RP18 (LiChrospher 100 CH-18/2), 250 mm×4 mm I.D., 5 μm particle size); mobile phase, 0.01 M potassium dihydrogenphosphate (pH 2.5 or 7.0); flow-rate, 1.0 ml/min; absorbance, 260 nm; detector sensitivity, 0.02 a.u.f.s. Peaks: 1 = cytosine; 2 = uracil; 3 = cytidine; 4 = uric acid; 5 = hypoxanthine; 6 = deoxycytidine; 7 = uridine; 8 = thymine.

Cyt, Cyd and Hyp rose slightly and those for Ura and Urd were unaffected. Optimal overall resolution for the pyrimidine compounds was obtained using pH 2.5, as illustrated in Fig. 1.

Calibration curves

To determine the response factor of the reference compounds (μmol per peak area), the linearity of the HPLC analysis of a known previously determined concentration was verified over the range of physiological interest. Calibration curves for pyrimidines, UA and Hyp concentrations were linear over the concentration range 0–40 μM. All correlation coefficients were between 0.99 and 1.00.

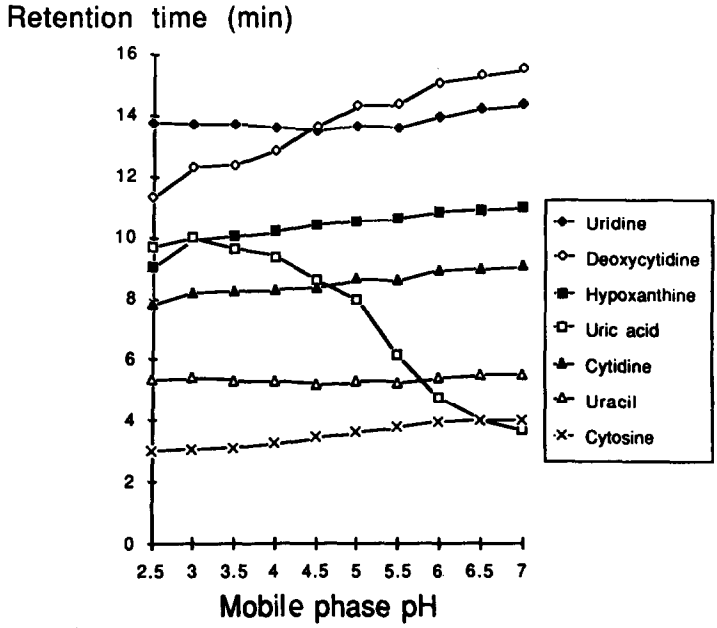

Fig. 2. Effect of mobile phase pH on HPLC retention times of bases and nucleosides tested. Chromatographic conditions were the same as in Fig. 1.

TABLE I

PYRIMIDINE BASE AND NUCLEOSIDE CONCENTRATIONS IN BLOOD PLASMA

Values are means ± S.D. of n (in parentheses) determinations.

Sample	Concentration (μM)				
	Uridine	Uracil	Cytidine	Cytosine	Deoxycytidine
Rat	1.15±0.38 (56)	1.67±0.4 (29)	5.35±0.95 (46)	1.09±0.43 (39)	41.0±8.0 (21)
Guinea pig	13.30±4.90 (8)	0.50±0.2 (13)	3.91±0.67 (9)	0.38±0.20 (13)	1.4±0.3 (8)
Rabbit	11.20±1.10 (6)	5.00±0.8 (6)	4.79±0.52 (6)	2.16±0.33 (6)	2.3±0.9 (6)
Dog	<3 (4)	0.71±0.2 (3)	1.01±0.50 (4)	0.90±0.30 (3)	26.0±5.0 (4)
Men	4.90±0.70 (9)	1.01±0.3 (5)	0.60±0.10 (4)	1.38±0.65 (9)	<0.5
Women	4.50±0.70 (6)	1.84±0.6 (6)	0.65±0.06 (6)	1.65±0.30 (6)	<0.5

Using the absorbance at 260 nm and 274 nm, the peak-area ratio 274/260 for each of these compounds was: Cyt, 1.79±0.03; Ura, 0.55±0.03; Cyt, 1.87±0.14; UA, 1.99±0.21; Hyp, 0.30±0.02; dCyd, 1.58±0.13; Urd, 0.49±0.04; Thy, 0.83±0.02 (mean ± S.D. of six to eight analyses).

Plasma concentrations

Table I gives the physiological concentration values of deproteinized plasma samples for several animal species. These data revealed great differences in the concentration of plasma compounds from one species to another. Ura and Cyt

concentrations lie in the range 0.5–2 μM, except for the rabbit in which Ura is appreciably more abundant. Thy was not significantly detected in any cases studied. Urd is present at a very low concentration in the dog, and in the other species the relative concentration is: rabbit > human > rat, with the ratio being roughly 12:4:1. The Cyd concentration in the rat, the guinea pig and the rabbit lies in the range 4–6 μM, whereas in the dog and the human it is ca. four to eight times less. The level of dCyd is almost undetectable in humans, is of the order of 1–2 μM in the guinea pig and the rabbit and is twenty to thirty times higher in the dog and the rat.

Analysis by HPLC of the radioactivity distribution in plasma

Table II summarizes the distribution of radioactivity among the pyrimidine derivatives in plasma 1 h after the intravenous injection of the labelled pyrimidine bases or nucleosides. HPLC eluates were collected in six fractions as described in Experimental. The sum of the radioactivity of these fractions corresponded to 96% of the radioactivity of the sample submitted to chromatography. The fraction of the total radioactivity present in the plasma remaining associated with the marker was 6% when labelled Ura was used as tracer, only 1% when the tracer was labelled Urd, 82% when the tracer was labelled Cyt and 44% when the tracer was labelled Cyd. When either Urd or Cyd was used as marker, their respective bases and the other blood pyrimidine nucleoside became labelled. In the cases of labelled Urd and Ura, a large part of the radioactivity was associated with compounds which eluted at the beginning of the HPLC chromatogram ($t_R < 3$ min).

In order to determine whether such a degradation of the marker takes place in blood, experiments were carried out in which labelled Urd or Cyd was added to blood or blood plasma samples of rats and then incubated at 37°C for 60 min (cf. Experimental). These experiments showed that in all cases the radioactivity present in acid-soluble extracts submitted to chromatography was to ca. 97% linked to their respective markers.

TABLE II

DISTRIBUTION OF THE RADIOACTIVITY AMONG PYRIMIDINE DERIVATIVES OF BLOOD PLASMA OF A SINGLE LABELLED DOSE OF URACIL, URIDINE, CYTOSINE OR CYTIDINE TO RATS

Values are means ± S.D. ($n = 4$ or 5), 1 h after intravenous administration.

Tracer	HPLC distribution as a percentage of the radioactivity of acid-soluble extract					
	Before cytosine	Cytosine	Uracil	Cytidine	Deoxycytidine	Uridine
[U-^{14}C]Uracil	85 ± 8	1.6 ± 0.3	6.0 ± 2.0	1.3 ± 0.4	1.5 ± 0.4	1.5 ± 0.3
[U-^{14}C]Uridine	88 ± 10	1.8 ± 0.4	1.2 ± 0.4	2.2 ± 0.8	< 0.5	1.2 ± 0.4
[2-^{14}C]Cytosine	11 ± 1.5	82.0 ± 1.0	< 0.5	< 0.5	< 0.5	< 0.5
[U-^{14}C]Cytidine	12 ± 2	28.0 ± 3.0	4.0 ± 2.0	44.0 ± 2.0	1.5 ± 0.3	6.0 ± 2.0

DISCUSSION

The intravenous injection of radioactive pyrimidine bases or nucleosides is one of the procedures used to study in situ the metabolism of pyrimidine nucleotides and RNA. To reach an accurate evaluation of the extent of incorporation of the tracer into a given tissue, measurement of the concentration and of the specific activity of the tracer in plasma is essential. The absence of a single procedure permitting the analysis of all pyrimidine bases and nucleosides of blood plasma led us to elaborate this HPLC method, which renders possible not only quantitative measurements but also the separate recovery of each component without degradation or radioactive contamination.

Blood collection and treatment of plasma

To obtain physiological concentrations of plasma compounds, blood must be obtained without haemolysis or coagulation occurring. Plasma must be quickly separated from blood cells and immediately deproteinized. These requirements are necessary to prevent bases and nucleosides from blood cells being subsequently released into the plasma. Indeed, the level of Urd in serum is higher than that in plasma [3], and the level of Hyp increases ten- to thirty-fold according to whether blood is stored at 4°C or at ambient temperature [15,16]. We also observed that the haemolysis of blood, even to a small extent, increases the concentration of most of the acid-soluble compounds of plasma. Another factor to be taken into account is the moment of blood collection, because of the circadian fluctuations of plasma nucleoside concentrations [3].

The deproteinization by perchloric acid and the neutralization of the acid supernatant by potassium hydroxide enables ca. 95% of the pyrimidine compounds to be recovered, in less than 30 min, ready for HPLC analysis. Because of its efficiency and reproducibility (controlled using labelled compounds, either isolated or added to plasma), this procedure can be used to obtain acid-soluble compounds of any biological fluid.

HPLC analysis

All pyrimidine compounds are eluted by our isocratic HPLC method in less than 18 min; however, the elution must be prolonged for ca. 60 min, so that the compounds eluted with long retention times do not contaminate subsequent chromatograms. Cyd, dCyd and Urd nucleosides are well resolved, so their accurate quantification is readily achieved and the recovery of the corresponding eluates causes no problems (see Experimental). Ura and Cyt bases are less well resolved, because of the great number of other compounds, as yet unidentified, that are eluted at similar times. In spite of this, the two bases can be quantified fairly accurately and their respective eluate fractions recovered without difficulty. However, the resolution of these bases can be improved, either by elution at pH 7.0 for Ura or by measurement of the absorbance at 274 nm for Cyt. For all the species studied the concentration of thymine in the blood plasma is too low to be quantified, despite the good separation obtained with standards. This confirms an earlier study in human plasma [13].

Chromatographic peaks were identified by (a) consideration of the retention times of standard compounds, (b) the addition of a standard mixture to plasma samples, (c) the determination of the absorption ratio at 260/280 nm and (d) the addition of radioisotopic markers.

Analysis with phosphate buffers of different molarities revealed no significant variation of the retention times of the pyrimidine compounds in the range 0.005–0.100 *M*. Nevertheless, with the type of stationary phase used in this study, the pH value of the mobile phase has an important influence [14,15,17,18]. In the pH range 3.5–6.5, the retention times of UA and dCyd vary significantly (Fig. 2), and thus these two compounds contaminate or mask one or several pyrimidine derivatives. Our evaluation of the UA concentration in plasma was 28 ± 9 μM ($n=20$) in rats, 15 ± 3 μM ($n=8$) in guinea pigs, 13 ± 2 μM ($n=6$) in rabbits, 15 ± 7 μM ($n=4$) in dogs, 159 ± 23 μM ($n=6$) in women and 277 ± 38 μM ($n=9$) in men; these values are in agreement with those reported in the literature [4,5,19]. This indicates that UA is one of the most abundant UV-absorbing acid-soluble compounds in plasma and is eluted with a retention time close to those of pyrimidine bases and nucleosides. Our results (Fig. 2) show that the retention times of the bases and nucleosides are in line with the classical concept, according to which compounds with pK values below 4.0 or above 8.0 show little variation in retention time with varying pH of mobile phase, whereas compounds with pK values between 4.0 and 8.0 show considerable variations [15,17,18].

Physiological pyrimidine concentrations

To demonstrate the potential application of this method to the quantification of the pyrimidine bases and nucleosides in biological fluids, deproteinized plasma samples from the various animal species were analysed. The physiological concentrations of pyrimidine bases and nucleosides in blood plasma reveal important quantitative and qualitative variations from one species to another (Table I). Such differences probably reflect the specificity of the metabolism of the nucleotides and RNA for each species. The fact that the liver and myocardium of the adult rat use more plasma Cyd than Urd for the synthesis of their pyrimidine nucleotides [8,9,20,21] is probably due to a greater abundance of Cyd in the plasma. Indeed, in these tissues the same enzyme (uridine-cytidine kinase) is responsible for the phosphorylation of both Urd and Cyd [22]. It is, therefore, reasonable to suppose that in the human metabolism, unlike that of the rat, Urd (present in greater amounts in the plasma than Cyd) would be the main precursor of nucleotides.

Radioactivity analysis

The radioactivity present in rat plasma following the intravenous injection of one of the four radioactive pyrimidine derivatives (Table II) is associated to the extent of ca. 95%, with the Ura, Urd, Cyt, Cyd and with the unidentified compounds eluted before Cyt. The degree of radioactivity of each compound depends on the nature of the marker injected. When Cyt and Cyd are the tracers, the greater part of the circulating radioactivity is associated with the tracer, whereas with Ura and Urd, most of the plasma radioactivity is associated with the un-

known compounds eluted at the beginning of the chromatogram. This indicates that Urd and Ura undergo very rapid metabolism. Since no degradation of Urd or Cyd was observed after incubation of either blood or blood plasma, there are reasonable grounds for supposing that the degradation of Urd and Ura, and Cyt, which is less appreciable, takes place in one of the organs or in the vascular tissues. Experiments were effected with hirudine as anticoagulating agent (the most specific inhibitor of the trombin), to prevent the possible secondary inhibiting effect of heparin on hypothetic blood enzymes, which could be responsible for the degradation of Urd and Ura. No differences were observed between the results of the hirudine and heparin experiments. The possibility of estimating the radioactivity associated with each pyrimidine compound, and the quantification of its plasma concentration, means that the specific activity of each compound in plasma can be calculated.

CONCLUSION

The speed of measurement of the physiological or pathological concentration of plasma pyrimidines, added to the possibility of collection of the compounds without any degradation or contamination, offers interesting prospects for studying the metabolism of the precursors of nucleotides and RNA. Furthermore, this method could also be used to diagnose particular illnesses or to investigate cases of toxicity caused by chemotherapy in which base or nucleoside levels in plasma are modified.

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