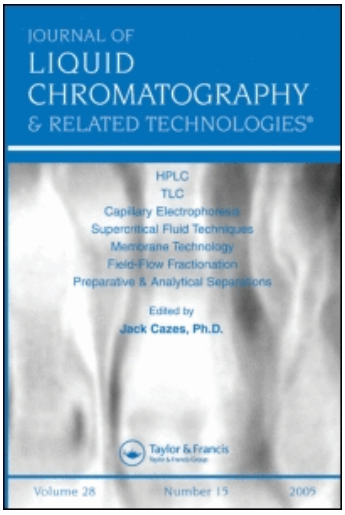

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THE APPLICATION OF UV-RADIOACTIVITY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TO THE STUDY OF HYPOXANTHINE TRANSPORT IN HUMAN ERYTHROCYTES

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ABSTRACT

A procedure is presented for the simultaneous measurement of concentrations of labeled and non labeled hypoxanthine by HPLC in order to study hypoxanthine transport in erythrocytes. A radioactivity detector connected on-line to the high performance liquid chromatograph in series with a UV detector provides on-line quantitative monitoring of hypoxanthine in erythrocytes or incubation medium. The procedure provides a rapid, sensitive and convenient means for the study of hypoxanthine transport.

INTRODUCTION

A rapid and selective reversed phase high performance liquid chromatographic method for the simultaneous determination of two important purine bases hypoxanthine and xanthine, in biological fluids has recently been developed in our laboratories (1). During analysis of hypoxanthine and xanthine in plasma and erythrocyte samples by this method, we observed an evolution of the hypoxanthine level in plasma samples during the time elapsed between sampling and centrifugation (2). This evolution would seem to be due to the increase of hypoxanthine in erythrocytes with time and at the release of

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hypoxanthine into the plasma. For these reasons, we set up a chromatographic system to study the transport of hypoxanthine in erythrocytes. This system consisted of a radioactivity detector connected on-line to the high performance liquid chromatograph in series with a UV detector.

The transport of hypoxanthine in erythrocytes has already been examined (3 - 8) however, the transport mechanism has not yet been clearly understood generally for technological reasons.

In the procedure presented here, the coupling of radioactivity and UV detectors makes possible the simultaneous measurement by direct means of the labeled and unlabeled hypoxanthine concentrations in the erythrocytes and the incubation medium. Furthermore given the sensitivity of the method, the studies can be carried out at physiological concentrations.

MATERIALS AND METHODS

Reagents

Potassium dihydrogenophosphate, sodium chloride, trichloracetic acid 20% were obtained from Merck (Darmstadt, GFR). Potassium chloride and glucose were obtained from Prolabo (Paris, France). Hypoxanthine was purchased from Sigma (St Louis, Mo, USA). (8 - ^{14}C) hypoxanthine (52 mCi/mmol) was obtained from CEA (F 91191, Gif sur Yvette, France). Liquid scintillator, Lumaflow II was obtained from Roche Kontron (Montigny le Bretonneux, France).

Saline medium : the composition of the saline medium was the following: 0.01 M sodium phosphate buffer, pH 7.40, KCl 5.6 mM, NaCl 153 mM and glucose 5mM.

Apparatus and Chromatographic Conditions

Chromatographic analyses were done with an integrated unit, a S P 8000 high performance liquid chromatograph (Spectra Physics, Orsay les Ullis, France). The column (15 cm x 4.6 mm I.D.) and a precolumn (5 cm x 4.6 mm I.D.) were packed with Hypersil ODS 3 µm (Shandon, Cheshire, Great Britain) by the slurry packing technique described by Coq et al (9). The mobile phase consisted of 0.02 M KH_2PO_4, the pH of which was adjusted to 3.65 with phosphoric acid. The flow rate was 1.5 ml/min. Detection was carried at 254 nm.

Radioactivity was measured using a Flo-One radioactive flow detector (Roche Kontron, Montigny le Bretonneux, France) connected on-line to the high performance liquid chromatograph in series with the UV detector, so that the column effluent first passed through the UV detector, then through the radioactivity detector where it was mixed with scintillator fluid before passing into the cell and out to waste.

The radioactivity detector is equipped with a 0.5 ml volume cell. The liquid scintillator used was Lumaflow II, the flow rate was 1.5 ml/min.

This apparatus was coupled to a 10 mV data recorder (Servotrace, Sefram, Paris, France). The Flow-One detector was used for the measurement of low energy, Beta-emitting radionuclides such as Carbon - 14. This instrument is equipped with a microprocessor which regulates the pump, operating programs and system controls. In the integrate mode, the peak elution times and total counts accumulated under the peak were printed out.

Blood Samples

Blood samples (5 to 10 ml) from different donors were collected in a heparinized tube and immediately centrifuged at 3000 r pm. Plasma, leucocytes and the upper layer of erythrocytes were removed.

The Time Course of Hypoxanthine Uptake by Erythrocytes

The time course of hypoxanthine uptake was measured at three different concentrations of $(8 - {}^{14}C)$ hypoxanthine : 10, 40, 80 $\mu mol.l^{-1}$. The assays were carried out with a Beckman microfuge using 0.4 ml polyethylene tubes. An aliquot of erythrocytes was suspended in an equal volume of saline medium preloaded with $(8 - {}^{14}C)$ hypoxanthine. The incubations were done at 25°C. At fixed times, the incubations were terminated by centrifuging the erythrocyte suspension. The $(8 - {}^{14}C)$ hypoxanthine taken up by the erythrocytes was determined by HPLC analysis after deproteinisation with trichloracetic acid 12%.

Hypoxanthine Distribution between Erythrocytes and Saline Medium at the Steady State

The saline medium was preloaded with $(8 - {}^{14}C)$ hypoxanthine at different concentrations : 0, 10, 20, 40, 80, 160 $\mu mol.l^{-1}$. The erythrocytes

from six different donors were incubated with the medium (1 vol/1vol) for five minutes at 25°C with gentle shaking, and the cells were separated from the saline medium by centrifugation. Both the erythrocytes and the saline medium were analysed by HPLC.

RESULTS AND DISCUSSION

Figure 1 shows the chromatograms from UV - radioactivity analysis of human erythrocytes (a) and saline medium (b) before incubation. The chomatograms of the analysis of the same sample after incubation are shown in figure 2. In this example the saline medium is preloaded with 10 $\mu mol.l^{-1}$ $(8 - {}^{14}C)$ hypoxanthine. Figure 2 shows that hypoxanthine crosses the cells membrane of erythrocytes. The radioactive peak follows the UV peak by a time factor which depends on the flow rate and volume between detector cells.

Linearity studies show that in radioactivity, the relation between the total counts measured under the peak and the concentration of $(8 - {}^{14}C)$ hypoxanthine is perfectly linear up to 40 $\mu mol.l^{-1}$. In UV, the linearity is excellent up to 50 $\mu mol.l^{-1}$.

The detection limit in UV for hypoxanthine is about 2.5 pmol. In radioactivity, the detection limit is four to five times lower, however at high sensitivities, the baseline is noisier in radioactivity than in UV detection.

The time course of the uptake of hypoxanthine by erythrocytes was measured at the three following concentrations : 10, 40 and 80 $\mu mol.l^{-1}$ $(8 - {}^{14}C)$ hypoxanthine. The incubations were carried out at 25°C and not at 37°C so as to allow the necessary time to carry out the study, the rate of uptake being less at lower temperatures (3).

Figure 3 shows that the equilibrium was reached within one to two minutes at 25°C which indicates that the uptake process is rapid at physiological concentrations of hypoxanthine.

During the radioactivity analysis we checked that no Inosine monophosphate peak was in fact present on the chromatograms. A hypoxanthine peak was the only radioactive compound found which proved that no metabolic conversion took place during the experiments.

The coupling of the radioactivity detector on-line with the UV detector made possible the simultaneous determination of the labeled and unlabeled

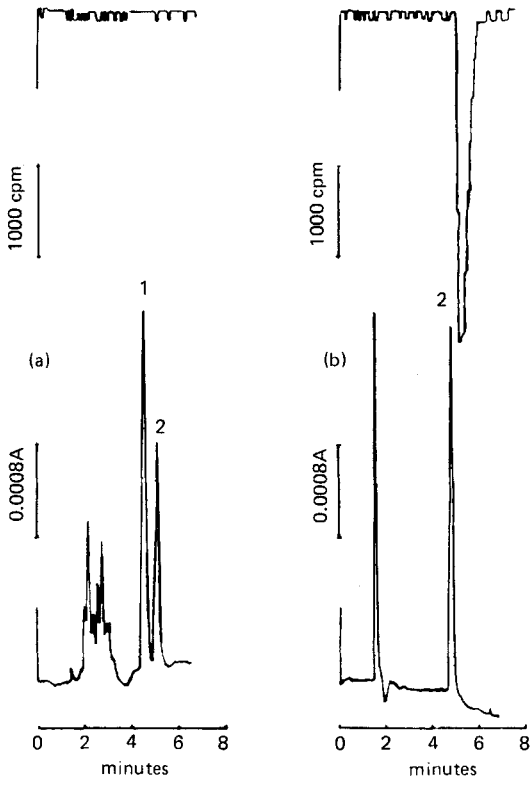

Figure 1 :

Chromatograms from the UV - radioactivity analysis of human erythrocytes (a) and of a saline medium (b) before incubation.

Analysis conditions : injection volume : 10 µl. Column : Hypersil ODS 3µm. Mobile phase : 0.02 M KH_2PO_4, pH 3.65 ; flow rate : 1.5 ml/min. UV detection : 254 nm. Liquid scintillator : Lumaflow II ; flow rate : 1.5 ml/min.

Peaks - 1 : uric acid, 2 : hypoxanthine (Hyp)

Radioactivity analysis : (a) Hyp labeled : 0 $\mu mol.l^{-1}$
(b) Hyp labeled : 10 $\mu mol.l^{-1}$

UV analysis (a) Hyp unlabeled : 4.5 $\mu mol.l^{-1}$
(b) Hyp labeled : 10 $\mu mol.l^{-1}$

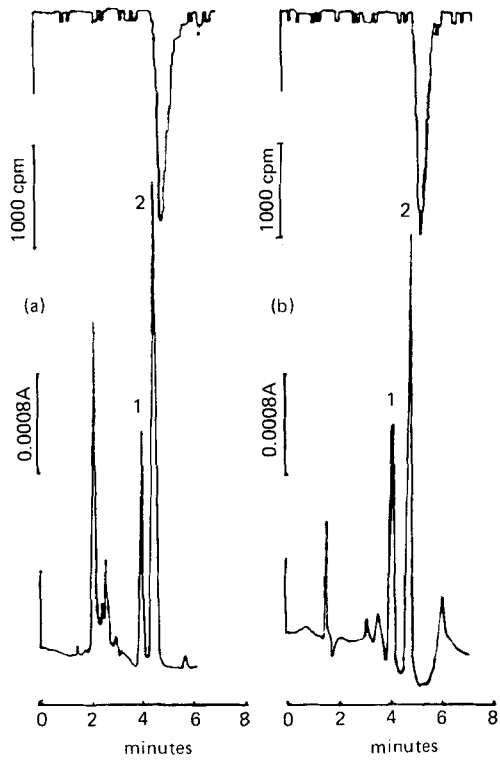

Figure 2 :

Chromatograms from the UV - radioactivity analysis of the same samples as in figure 1, erythrocytes (a), saline medium (b), but after incubation.

Peaks - 1 : uric acid, 2 : hypoxanthine

Radioactivity analysis : (a) Hyp labeled : 4.8 $\mu mol.l^{-1}$
(b) Hyp labeled : 5.0 $\mu mol.l^{-1}$

UV analysis (a) Hyp (labeled + unlabeled) : 7.2 $\mu mol.l^{-1}$
(b) Hyp (labeled + unlabeled) : 7.1 $\mu mol.l^{-1}$

hypoxanthine concentrations in both erythrocytes and saline medium after incubation. Using this procedure, we considered the distribution of labeled and unlabeled hypoxanthine between erythrocytes and saline medium at the steady state. An example of this hypoxanthine distribution between erythrocytes of a healthy subject and saline medium loaded with (8 - ^{14}C) hypoxan-

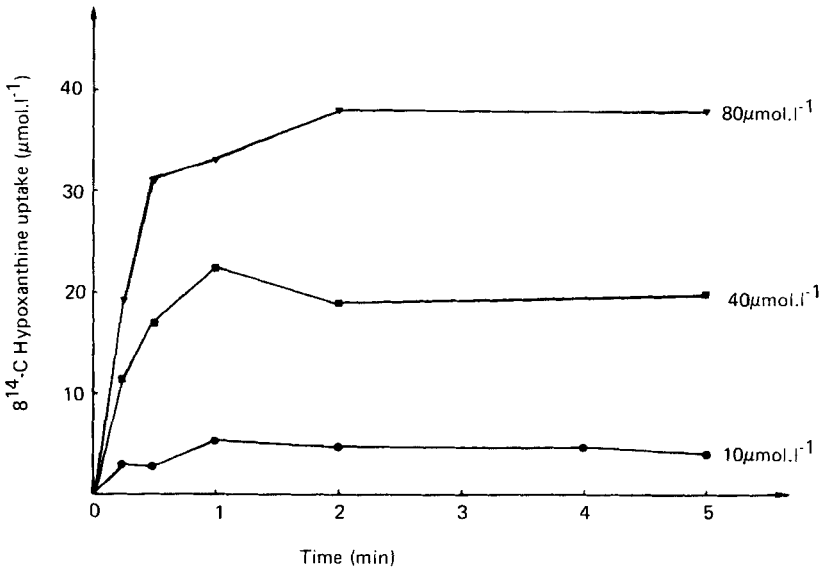

Figure 3 :

The time course of hypoxanthine uptake by human erythrocytes from 10, 40, 80µmol.l^{-1} (8 - ^{14}C) hypoxanthine in the incubation medium.

TABLE 1

Distribution of Labeled and Unlabeled Hypoxanthine between the Erythrocytes of a Healthy Subject and a Saline Medium after Incubation

Erythrocytes		Saline medium	
Hyp	8 - ^{14}C	8 - ^{14}C	Hyp
9	0	0	8
8.5	4.8	5.0	9
9	10	9.0	8
9	20	19.5	7.5
9	39	39.0	8
7.5	72.5	90.0	9.0

thine at different concentrations ranging from 0 to 160 $\mu mol.l^{-1}$ is presented in table 1. This table indicates that after incubation at 25°C for five minutes both labeled and unlabeled hypoxanthine reach approximately equal concentrations in the cells and in the medium. The results obtained from the experiments on erythrocytes of five healthy subjects are similar.

Conditions of the experiment :

- Initial Hyp concentration in erythrocytes : 17 $\mu mol.l^{-1}$
- Initial labeled Hyp concentrations in saline medium : 0, 10, 20, 40, 80, 160 $\mu mol.l^{-1}$.
- Incubation : 5 minutes at 25°C.

The data show that hypoxanthine crosses the cell membrane of erythrocytes in both directions : the hypoxanthine in the medium is partially taken up by the erythrocytes, and the hypoxanthine initially present in the erythrocytes is partially released into the medium. These observations lead us to conclude that the increase of hypoxanthine concentration in plasma samples left in contact with erythrocytes may in fact be due to a hypoxanthine release from the erythrocytes into the plasma.

As a more general conclusion, we feel that the system described in this paper, may indeed be one of the most suitable for the monitoring of labeled and unlabeled compounds in transport studies.

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