Journal of Chromatography, 491 (1989) 468–472
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4763

Note

High-performance liquid chromatographic determination of rhodamine B in rabbit and human plasma

R.W. MASON* and I.R. EDWARDS

Toxicology Research Unit (Medical Research Council of New Zealand), University of Otago Medical School, P O. Box 913, Dunedin (New Zealand)

(First received December 28th, 1988; revised manuscript received March 9th, 1989)

Rhodamine B, 9-(2-carboxyphenyl)-3,6-bis(diethylamino)xanthylium chloride, is widely used as a fabric dye, a colouring agent in drug and cosmetic preparations, an analytical reagent for metals, a tracing agent in water pollution studies and as a colour marker in herbicide sprays [1]. Animal studies have shown that rhodamine B may induce growth retardation and liver damage [2], erythrocyte haemolysis [3] and the suppression of the immune response in isolated spleen cells [4]. The dye has also been recognised as possibly mutagenic [5–9] and carcinogenic [10,11]. Apart from the work of Webb and Hansen [12,13], who have studied the metabolism of rhodamine B in the dog, rat and rabbit, there is little information available on the pharmacokinetics of rhodamine B in either experimental animals or in man. We report here the development of a high-performance liquid chromatographic (HPLC) method for the quantitation of rhodamine B in rabbit and human plasma. The method is tested by the analysis of plasma following the treatment of rabbits with rhodamine B.

EXPERIMENTAL

Chemicals and reagents

A commercial preparation of rhodamine B (rhodamine B500 powder) was provided by ICI New Zealand (Wellington, New Zealand). Analytical-grade

0378-4347/89/$03.50

rhodamine B was supplied by Solmedia (London, U.K.). Acetonitrile (HPLC grade) was obtained from J.T. Baker or May and Baker (Dagenham, U.K.) and orthophosphoric acid (Aristar grade) from BDH (Poole, U.K.). Distilled, deionised water was obtained using the Millipore Q system, whilst all other reagents were of analytical grade.

Analytical procedures

The detection parameters for rhodamine B were established following the determination of the fluorescence spectrum of the dye in aqueous solution using a Shimadzu DR-3 spectrofluorimeter. Analyses were carried out at room temperature ($18 \pm 2°C$) using a μBondapak CN column (25 mm$\times$4.6 mm) with a Waters 6000A pump, a U6K injector and a Schoeffel FS 970 fluorimetric detector. The mobile phase consisted of acetonitrile–water (35:65 or 40:60) containing 0.1% orthophosphoric acid at a flow-rate of 1.8 ml/min. Chromatograms were recorded with a chart-speed of 2.5 mm/min. Samples of plasma (0.5 ml, diluted with 0.5 ml of 0.05 *M* potassium dihydrogenphosphate buffer, pH 5.5) were extracted with 5 ml of ethyl acetate, the organic phase (4 ml) was evaporated to dryness, the residue dissolved in 250 μl of the mobile phase and a small volume (5–50 μl) of this solution injected into the liquid chromatograph. Standard calibration curves were obtained by analysing plasma containing known amounts of rhodamine B. Validation experiments were carried out to define the linearity of the relationship between peak height and concentration and to determine the extraction efficiency and reproducibility of the analytical procedure.

Pharmacokinetics of rhodamine B in the rabbit

Adult female New Zealand White rabbits (3–4 kg) were given rhodamine B500 (1 mg/kg) by intravenous injection into the ear vein. Blood samples (1 ml) were collected from the central artery of the opposite ear at various times up to 300 min following the administration of rhodamine B500. The plasma was analysed as described above and the rhodamine B concentration calculated by comparing the observed peak height with that obtained from a standard solution containing a known amount of rhodamine B.

RESULTS AND DISCUSSION

Rhodamine B was strongly fluorescent, giving excitation and emission maxima in aqueous solution at 261 and 575 nm, respectively. Thus, an excitation wavelength of 260 nm together with an emission filter at 580 nm were chosen as appropriate detection parameters. The assay provided a good separation of rhodamine B from several other fluorescent compounds which were present in the plasma of rhodamine B500-treated rabbits but were absent from the plasma of untreated rabbits (Fig. 1). The chromatographic conditions of the assay

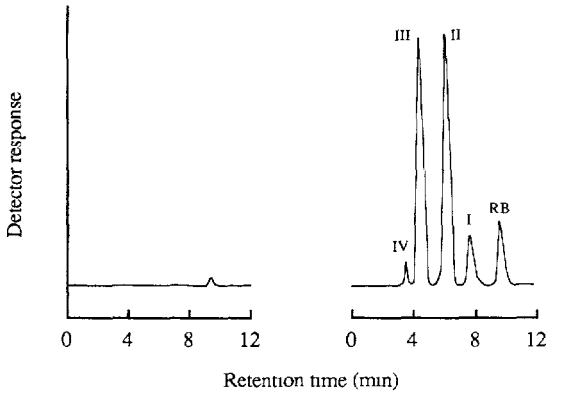

Fig. 1. HPLC profiles of plasma extracts before treatment and 2 h after treatment of a female rabbit with 1 mg/kg rhodamine B500 intravenously. Peaks: RB = rhodamine B; I–IV = metabolites I–IV, respectively.

were chosen to optimise the analysis of rhodamine B at the expense of its metabolites. Under the conditions of the assay, four main fluorescent components were resolved with retention times of 7.6 min (metabolite I), 6.0 min (metabolite II), 4.5 min (metabolite III) and 3.7 min (metabolite IV). The retention time of rhodamine B was 9.7 min and the total analysis time approximately 12 min. The elution of the solute components may be assumed to have occurred in order of decreasing polarity and water solubility. Thus the results of Fig. 1, which show that the fluorescent metabolites chromatographed with faster retention times than the parent compound, are consistent with the observations of Webb and Hansen [12], who demonstrated that in the dog, rat and rabbit rhodamine B is metabolised by step-wise de-ethylation to N,N′-diethyl-3,6-diaminofluoran, the monoethyl analogue and 3,6-diaminofluoran, with each compound being more water-soluble than its precursor.

Fluorescein, fluorosceinamine, fluoroscein diacetate, tetraiodofluoroescein, tetrabromofluoroscein, diiododimethylfluoroscein, anthraquinone-2-sulphonic acid and phyloxine were tested as possible internal standards for the analysis but none of these compounds were suitable because their retention times coincided with those of the metabolite peaks.

Calibration curves for rhodamine B in rabbit plasma were linear at concentrations ranging from 25 to 500 ng/ml. A typical analysis gave a correlation coefficient (r) of 0.9947 ($n=6$) with recoveries and coefficients of variation (C.V.) of $98 \pm 5\%$ (mean $\pm$ S.D., $n=5$) and 5.3%, respectively, at the highest concentration and $87 \pm 10\%$ and 11%, respectively, at the lowest concentration. The calibration curve for rhodamine B in human plasma was also linear over the same range of concentrations. A typical standard curve ($r=0.9995$, $n=6$) gave an overall recovery of $106 \pm 9\%$ ($n=5$) and a C.V. of 10.8% at the lowest concentration and 3.6% at the highest concentration. The detection

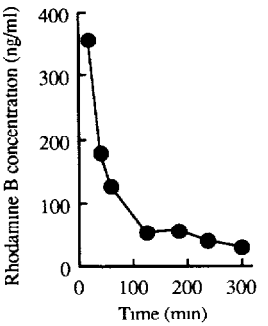

Fig. 2. Plasma concentration of rhodamine B as a function of time following the intravenous administration of 1 mg/kg rhodamine B500 to a female rabbit.

limit was at least 2 ng of rhodamine B, corresponding to a plasma concentration of 25 ng/ml, at a signal-to-noise ratio of 4:1.

The stability of rhodamine B in whole blood was assessed by treating a rabbit with rhodamine B, obtaining a blood sample after 3 h and then analysing the plasma immediately and after storage of the blood at room temperature for 1 h. During this period, the plasma concentration of rhodamine B remained constant.

The analytical method was tested in rabbits by analysing plasma following the intravenous administration of rhodamine B (Fig. 2). A representative plasma concentration–time curve obtained from one of the animals indicates that the proposed method is applicable in vivo. It is important to note the rapid decrease in the plasma concentration of rhodamine B (Fig. 2) and the rapid appearance of its metabolites in the plasma (Fig. 1). The significance of these observations and the role of the metabolites in the pharmacokinetics of rhodamine B require to be elucidated. Application of the present method to a study of the pharmacokinetics and genotoxicity of rhodamine B and its metabolites will be reported elsewhere.

REFERENCES

1 IARC Monographs, 16 (1978) 221.
2 W.H. Hansen, O.G. Fitzugh and M.W. Williams, J. Pharmacol. Exp. Ther., 122 (1957) 29A.
3 S.D. Gangolli, P. Grasso and L. Golberg, Food Cosmet. Toxicol., 5 (1967) 601.
4 J.A. Wess and D.L. Archer, Food Chem. Toxicol., 20 (1982) 9.
5 Z. Landa, P. Klouda and D. Pleskotova, in J. Veleminsky and T. Gichner (Editors), Induction of Mutations and the Mutation Process, Publishing House of the Czechoslavak Academy of Sciences, Prague, 1965, pp. 115–122.
6 I.L. Lewis, R.M. Patterson and H.C. McBay, Mutat. Res., 88 (1981) 211.
7 E.R. Nestmann, G.R. Douglas, T.I. Matula, C.E. Grant and D.J. Kowbel, Cancer Res., 39 (1979) 4412.
8 E.R. Nestmann, D.J. Kowbell and J. Ellentoin, Water Res., 14 (1980) 901.

9 J.P. Brown, P.S. Dietrich and C.M. Backner, Mutat. Res., 55 (1979) 181.
10 M. Umeda, Gann, 47 (1956) 51.
11 G.M. Bonser, B.B. Clayson and J.W. Jull, Br. J. Cancer, 10 (1956) 653.
12 J.M. Webb and W.H. Hansen, Toxicol. Appl. Pharmacol., 3 (1961) 86.
13 J.M. Webb and W.H. Hansen, Toxicol. Appl. Pharmacol., 3 (1961) 696.