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Note

High-performance liquid chromatographic determination of β-naphthoflavone in biological fluids

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β-Naphthoflavone (BNF) is a synthetic derivative of a large class of naturally occurring flavonoid compounds. A property of this group of compounds is their ability to induce the activity of hepatic microsomal monooxygenase enzymes [1]. Their importance in induction studies stems from the fact that they induce a different pattern of isozymes to that of drugs, like phenobarbitone [1–3]. BNF has an advantage over other inducers in this class, like 3-methylcholanthrene, with similar inductive activity in that it is non-carcinogenic [4–6]. BNF is one of the most potent of the polycyclic aromatic hydrocarbon class of inducers [6,7].

Despite wide usage, very little is known about the disposition of flavonoids. A recent study [8] has identified some hydroxylated metabolites of α-naphthoflavone and β-naphthoflavone from microsomes and purified cytochrome P-450 systems. Evidence has been produced to show that flavonoids are present as pollutants in the environment and thereby affect living systems [9]. There is no published assay method for their determination in biological fluids to allow the study of their disposition in animals. The methods available for the determination of polycyclic aromatic hydrocarbons in the environment [10–12] are not particularly suitable for their determination in biological fluids.

As part of a wider study of the effects of BNF on hepatic microsomal monooxygenase systems, we have developed a method for the determination of BNF in plasma and urine. A preliminary pharmacokinetic study of BNF was also carried out in the anaesthetised rat.

EXPERIMENTAL

Equipment and chemicals

The chromatographic equipment consisted of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A pump, a Pye-Unicam (Cambridge, U.K.) LC 3 UV vari-

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able-wavelength UV detector, a Model 7125 Rheodyne valve injector and a Phillips (Eindhoven, The Netherlands) PM 8251 single-pen recorder.

BNF and bovine serum albumin (BSA) fraction V powder were obtained from Sigma (Poole, U.K.). Isopropyl antipyrine was obtained from OPG (Utrecht, The Netherlands). Dichloromethane, AR grade (Fisons, Loughborough, U.K.), acetonitrile, HPLC grade, (Rathburn, Walkerburn, U.K.) and sodium acetate trihydrate, AR grade (BDH, Poole, U.K.) were all used without further purification.

Chromatography

The column was a commercially packed (Hichrom, Reading, U.K.) reversed-phase column (Zorbax TMS, 5 μm, 25 cm $\times$ 4.9 mm I.D.) through which a mobile phase consisting of 50% methanol in 0.2 *M* acetate buffer (pH 4.5) was run at a flow-rate of 1.0 ml/min and the eluent monitored at 275 nm. The column was washed after each day's use, first with water and then with methanol, for about 20–30 min each time.

Extraction from biological fluids

The extraction procedure involves the addition to a plasma (100 μl) or urine (500 μl) sample of an aqueous solution of isopropyl antipyrine (100 μl of 10 μg/ml) as internal standard and dichloromethane (5 ml). This was extracted for 10 min on a rotary mixer, centrifuged at 2000 *g* for 5 min and the aqueous layer was removed by aspiration. The organic layer was evaporated to dryness on a warm water-bath under a stream of air. The residue was kept frozen before analysis, then reconstituted in the mobile phase (100 μl) and 20 μl were injected onto the column. BSA solution (40 g/l) and various buffer solutions were assessed as alternatives to plasma for use in preliminary validation work on the assay and for preparing subsequent standards for calibration.

Assay validation

Solutions of various concentrations of BNF up to 100 mg/l were prepared and analysed to test assay linearity. The intra-day and inter-day variabilities of the assay were measured by preparing different concentrations within the linear region and carrying out replicate analyses both within the same day and on different days. The coefficient of variation associated with the results was used to assess reproducibility. The desirability of using internal standard was tested by assessing variabilities both with and without the internal standard.

Pharmacokinetic experiments in the rat

Male Sprague–Dawley rats (200–250 g) were anaesthetised with urethane (1200 mg/kg) and cannulated in the jugular vein and carotid artery. BNF (20 mg/kg) was administered as a solution (polyethylene glycol 400–propylene glycol, 9:1, v/v; 2 ml/kg) via the jugular vein. Serial blood samples (250 μl) were taken into heparinised tubes via the carotid artery over 4 h from which plasma (100 μl) was obtained by centrifugation (2000 *g* for 10 min) and analysed for BNF.

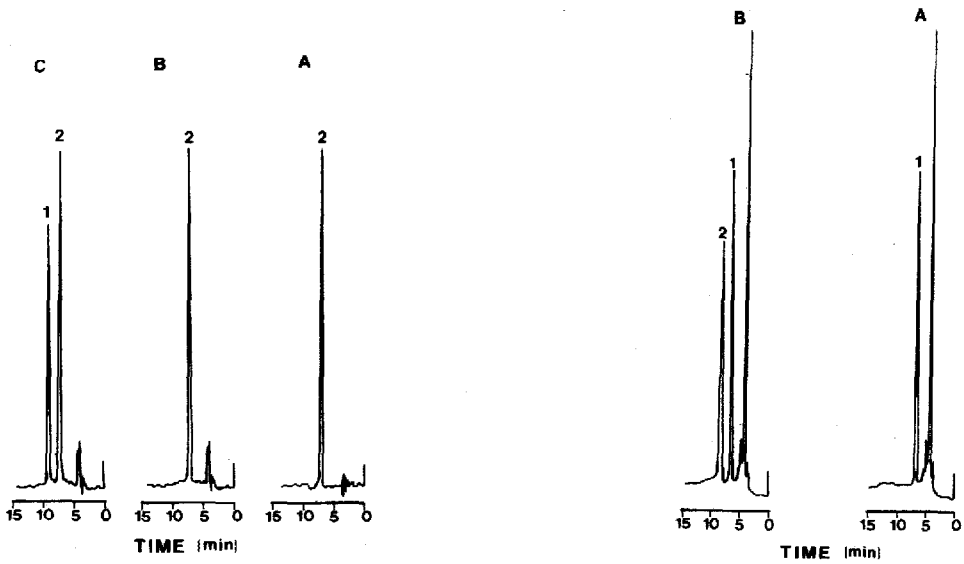

Fig. 1. Chromatograms of blank BSA (A), blank rat plasma (B) and plasma sample containing BNF (C). Peaks: 1 = BNF (3.5 mg/l); 2 = isopropyl antipyrine, internal standard (10 mg/l).

Fig. 2. Chromatograms of blank urine (A) and urine sample containing BNF (B). Peaks: 1 = isopropyl antipyrine, internal standard (10 mg/l); 2 = BNF (2.0 mg/l).

RESULTS AND DISCUSSION

The chromatograms obtained showed that there were no interfering peaks from either plasma, albumin solution (Fig. 1) or urine (Fig. 2) with the peaks of interest. Chromatographic time was less than 10 min with good resolution. The retention times were 9 min for BNF and 7 min for the internal standard.

The standard curve was found to be linear up to 50 mg/l for BNF in plasma or albumin solution and 30 mg/l for BNF in urine. BNF could be detected down to 0.1 mg/l in plasma or albumin solution and 0.05 mg/l in urine. In plasma or albumin solution above 50 mg/l, non-linearity occurs which appears to be due to

TABLE I

EXTRACTION AND ASSAY CHARACTERISTICS OF β-NAPHTHOFLAVONE FROM PLASMA, ALBUMIN SOLUTION AND URINE

Average extraction efficiency calculated by comparison with solutions made up in mobile phase over the entire linear concentration range. Concentrations were determined by use of peak-height ratios. $n = 8$.

Sample	Extraction efficiency (mean ± S.D.) (%)	Limit of linearity (mg/l)	Limit of detection (mg/l)
Plasma	79 ± 7	50	0.1
Albumin	81 ± 5	50	0.1
Urine	76 ± 19	30	0.05

TABLE II

INTRA-DAY AND INTER-DAY PRECISION OF HPLC ANALYSIS OF BNF

H = peak height alone; HR = peak-height ratio to internal standard; $n = 6$ for intra-day and $n = 4$ for inter-day comparisons.

Concentration (mg/l)	Coefficient of variation (%)			
	Intra-day		Inter-day	
	H	HR	H	HR
1.0	9.0	3.9	8.9	4.9
10.0	6.1	2.3	7.7	2.6
50.0	7.6	2.6	9.7	2.4

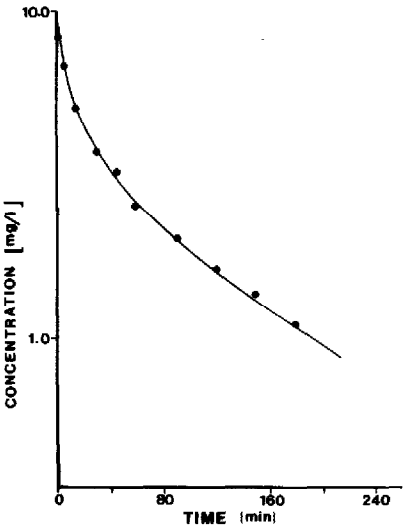

Fig. 3. Plasma concentration-time profile of BNF after administration of 20 mg/kg BNF intravenously to an anaesthetised rat. Each point is an average from two rats.

saturation of the extraction process. Results are shown in Table I. BSA (40 g/l) has extraction characteristics similar to plasma (Table I) and was closest to plasma of all solutions tested. It was therefore used for the validation and preliminary work on the assay and was also used for subsequent preparation of standards. The assay also shows an extraction efficiency of approximately 80% for BNF in plasma or albumin and urine (Table I).

The precision of the assay is shown in Table II. The coefficient of variation was less than 10% in all cases for both intra- and inter-day. The inclusion of an internal standard improved the reproducibility of the assay with a reduction in the associated coefficient of variation. The internal standard has no effect on the extraction characteristics of BNF from plasma or albumin solution.

The result of the preliminary pharmacokinetic experiments in the rat is shown in Fig. 3. BNF exhibits a biexponential disposition with an initial half-life of

about 7–10 min and a terminal half-life of about 80 min. It has a high clearance, 50 ml/min/kg, and a large volume of distribution, 6 l/kg.

CONCLUSION

A simple, rapid and reproducible assay has been developed for the determination of BNF in plasma and urine. This is the first report to our knowledge of an assay system for BNF in biological fluids. This method will therefore aid in the study on the disposition of BNF itself and in trying to relate its disposition to its inductive effect on hepatic microsomal monooxygenases. The similarity in the extraction characteristics of rat plasma and BSA (40 g/l) in the determination of BNF will allow BSA to be used in place of plasma for work on the assay thus avoiding the use of a large volume of blank rat plasma.

REFERENCES

1 F.P. Guengerich, G.A. Dannan, S.T. Wright, M.V. Martin and L.S. Kaminsky, Biochemistry, 21 (1982) 6019.
2 D.E. Williams and D.R. Buhler, Comp. Biochem. Physiol., 75C (1983) 25.
3 A. Aldridge and A.H. Neims, Drug Metab. Dispos., 7 (1979) 378.
4 P.P. Lau, C.B. Pickett, A.Y.H. Lu and H.W. Strobel, Arch. Biochem. Biophys., 218 (1982) 472.
5 L.W. Wattenberg, J.L. Leong and A.R. Galbraith, Proc. Soc. Exp. Biol. Med., 127 (1968) 467.
6 L.W. Wattenberg, M.A. Page and J.L. Leong, Cancer Res., 28 (1968) 934.
7 K.R. Cutroneo, R.A. Seibert and E. Bresnick, Biochem. Pharmacol., 21 (1972) 937.
8 K.P. Vyas, T. Shibata, R.J. Highet, H.J. Yeh, P.E. Thomas, D.E. Ryan, W. Levin and D.M. Jerina, J. Biol. Chem. 258 (1983) 5649.
9 G.L. Foureman, N.B. White, Jr. and J.R. Bend, Can. J. Fish Aquat. Sci., 40 (1983) 854.
10 E.P. Lankmayr and K. Müller, J. Chromatogr., 170 (1979) 139.
11 T. Nielsen, J. Chromatogr., 170 (1979) 147.
12 N.T. Crosby, D.C. Hunt, L.A. Phillip and I. Patel, Analyst, 106 (1981) 135.