ELSEVIER

Journal of Chromatography B, 732 (1999) 349–356

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Application of the high-performance liquid chromatographic method for separation, purification and characterisation of *p*-bromophenylacetylurea and its metabolites

Jinsheng Xu[a,*], John H. Lamb[b], Rebecah Jukes[b], Wendy M. Purcell[a], David E. Ray[b]

[a]*Department of Biological and Biomedical Sciences, Faculty of Applied Sciences, University of the West of England, Bristol, Coldharbour Lane, Bristol BS16 1QY, UK*

[b]*Medical Research Council Toxicology Unit, University of Leicester, Leicester, UK*

Received 26 March 1999; received in revised form 1 June 1999; accepted 22 June 1999

Abstract

This study presents a HPLC method for the separation and purification of *p*-bromophenylacetylurea (BPAU) and its metabolites. The method effectively separated and purified BPAU and its metabolites. Three metabolites of BPAU, M1, M2 and M3 were characterised by mass spectroscopy and nuclear magnetic resonance. They are named as *N′*-hydroxy-*p*-bromophenylacetylurea, 4-(4-bromophenyl)-3-oxapyrrolidine-2,5-dione and *N′*-methyl-*p*-bromophenylacetylurea, respectively. The major metabolic pathways of BPAU were proposed. The establishment of the HPLC method and characterisation of BPAU metabolites make it possible for further pharmacokinetic studies to explore the mechanism of BPAU-induced delayed neuropathy.

Keywords: *p*-Bromophenylacetylurea; *N′*-Hydroxy-*p*-bromophenylacetylurea; 4-(4-Bromophenyl)-3-oxapyrrolidine-2,5-dione; *N′*-Methyl-*p*-bromophenylacetylurea

1. Introduction

p-Bromophenylacetylurea (BPAU) was originally synthesised as one of a series of halogenated congeners of phenylacetylurea (Phenurone), an antiepileptic agent, to search for a more effective anticonvulsive drug [1]. Although its anticonvulsive effect was better than other derivatives of phenylacetylurea, it produced serious delayed ataxia and paralysis in rats [1,2]. Therefore, BPAU has been widely used as a model compound in mechanistic studies of delayed neuropathy in rats [2–4]. The mechanism of BPAU-induced neurotoxicity is not yet known. So far, intensive studies have been done on the clinical features, histopathological and electrophysiological aspects of BPAU-induced delayed neuropathy [1–10]. However, the metabolism and pharmacokinetics of BPAU has not yet been studied. The pharmacokinetics of BPAU is important in understanding its pharmacodynamic effect. To perform such studies, an analytical method is needed. The present study developed a high-performance

*Corresponding author. Tel.: +44-117-9656-261, ext. 3829; fax: +44-117-9763-871.

E-mail address: Jinsheng.Xu@uwe.ac.uk (J. Xu)

0378-4347/99/$ – see front matter
PII: S0378-4347(99)00293-5

liquid chromatographic method for the isolation and purification of BPAU and its metabolites and characterised in vivo metabolism of BPAU in the rat.

2. Materials and methods

2.1. Chemicals

BPAU was synthesised by Dr. R. Mattocks (MRC Toxicology Unit, Leicester, UK); acetonitrile (HPLC grade) and diethyl ether (HPLC grade) were purchased from Sigma. Dimethyl sulphoxide (DMSO) was purchased from BDH. Methanol and ammonium acetate were obtained from Fisons.

2.2. Animal and procedures

Four male Fisher 344 rats, body weight 240–250 g, were given 150 mg/kg BPAU in DMSO by gavage. Two rats were housed in metabolic cages with free access to food and water. Urine was collected between 12 and 48 h after dosing. The other two rats were housed in a plastic cage with a stainless steel top to allow access for blood and tissue collection. A control rat was given the same volume of DMSO (solvent) as test animals.

2.3. Sample preparation

The rats for blood and tissue collection were killed by carbon dioxide 24 h after dosing. Blood samples were collected through the heart immediately after death and allowed to clot. Each liver was dissected, weighed and stored at −20°C. Before analysis, livers were thawed and homogenised in methanol–DMSO (95:5, v/v) solution at 100 mg tissue per ml solution. Blood was centrifuged at 6000 *g* for 10 min and serum was retained and mixed with an equal volume of methanol–DMSO solution. In addition, five samples of fresh serum from the control rat were spiked with 5 μg/ml BPAU to study the recovery of the analytical method. These samples were also mixed with methanol–DMSO solution (1:4, v/v) as stated above. All samples were centrifuged at 9000 *g* for 5 min. The supernatants were retained and stored at −20°C for up to four weeks.

2.4. High-performance liquid chromatography (HPLC) analytical conditions

A Waters HPLC system with a Controller 600, a tuneable absorbency detector 486, and an autosampler 717 plus was employed. The column was a reversed-phase ODS2, S5, 250×4.6 mm (Spherisorb). Detector wavelength was 240 nm. The gradient of mobile phase (A=0.1 *M* ammonium acetate, pH=6.5; B=acetonitrile) was as follows: 25% B linear to 50% B within 10 min and then maintaining 50% B for 10 min. The flow-rate was 1 ml/min. The injected volume of each sample was 25 or 50 μl for quantitative assays and 100–200 μl for purification.

2.5. Metabolite purification

The HPLC profiles of BPAU and its metabolites are shown in Fig. 1. Three metabolites were temporarily designated M1, M2 and M3, respectively according to their first identification order. In order to characterise the metabolites, they were purified. Liver was used for M1 purification, serum and urine were used for M2 purification and M3 was purified from urine.

2.6. Pre-column extraction

2.6.1. M1

Liver was thawed and homogenised in a solution of methanol–DMSO (95:5, v/v) at 100 mg tissue per ml solution. The homogenate was centrifuged at 3000 *g* for 20 min. The supernatant was concentrated under reduced pressure with a Rotovaper-R in 37°C water bath for 40 min to evaporate methanol and stored at 4°C overnight, and then centrifuged (9000 *g*, 5 min, 4°C) to remove insoluble residue. The supernatant was retained and diluted with an equal volume of distilled water and then extracted three times with an equal volume of diethyl ether. The extract was concentrated again by evaporating diethyl ether with a Rotovaper-R for 30 min and the residue diluted with an equal volume of 25% acetonitrile. The solution was stored at 4°C overnight, centrifuged (9000 *g* for 5 min) and the supernatant reserved for purification by HPLC.

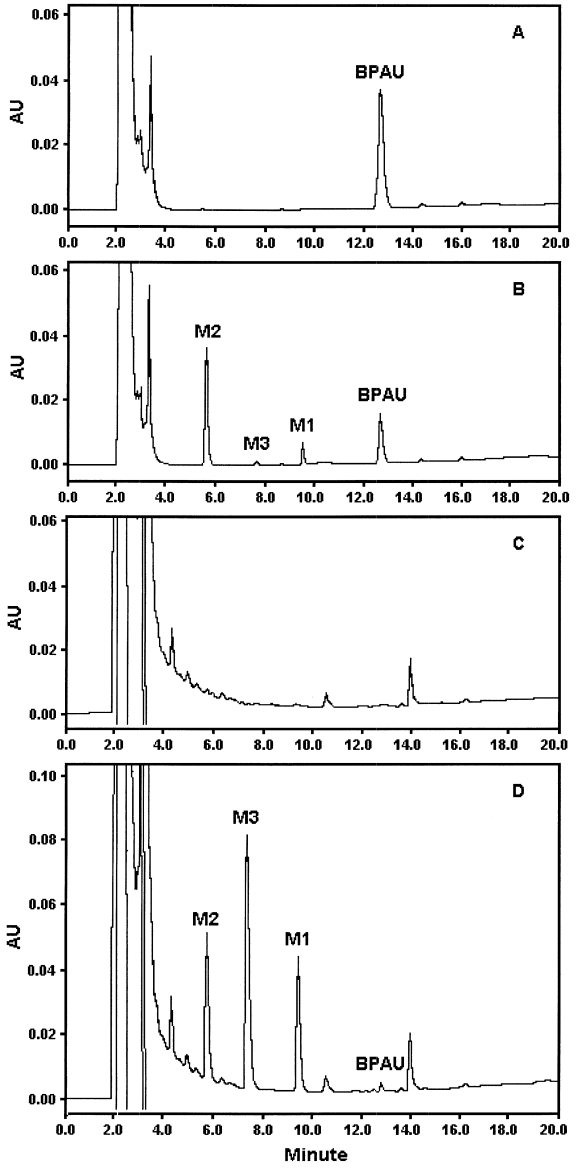

Fig. 1. HPLC profiles of BPAU and its metabolites, M1, M2 and M3, in serum and urine. (A) BPAU standard spiked with serum. (B) Serum sample 6 h after BPAU dosing. (C) Urine blank control. (D) Urine sample 24 h after BPAU dosing.

2.6.2. M2

Serum was mixed with methanol–DMSO (1:5, v/v) and centrifuged at 3000 *g* for 20 min. Further extraction and purification procedures were as stated above for M1.

2.6.3. M3

All the collected urine was pooled and centrifuged (3000 *g*, 20 min) and the supernatant retained and extracted three times with an equal volume of diethyl ether. Further extraction and purification procedures were as stated above for M1.

2.7. Purification

2.7.1. M1 and M3

The HPLC peak fractions of either M1 or M3 were collected. The collected solutions were each extracted three times with an equal volume of diethyl ether. The extraction was concentrated by evaporating the diethyl ether with a Rotovaper-R at room temperature for 30–40 min. The remainder was dissolved in 5–10 ml of 25% acetonitrile and purified for a second time by HPLC. The peak fraction of either M1 or M3 was collected and extracted again, as described above. The extraction residue from the second HPLC purification was placed in a clean glass test tube and evaporated under a gentle stream of nitrogen.

2.7.2. M2

The HPLC peak fraction of M2 was collected and diluted with 0.1 *M* ammonium acetate (1:1, v/v) and filtered through a cartridge (Bond Elut C_{18}, Varian). The cartridge was washed with 2 ml deionised water. M2 was then washed off with 5 ml methanol and collected in a clean glass test tube. The collected effluent was then dried under a gentle stream of nitrogen.

2.8. Stability

Purified M1, M2 and M3 were re-examined with HPLC. Their retention times were the same as in test samples.

2.9. Mass spectrum

Mass spectra of BPAU and purified M1, M2 and M3 were obtained on a V.G.70-SEQ instrument (VG Analytical, Manchester, UK) of EBqQ geometry. All samples were ionised by negative ion fast atom bombardment (FAB) using argon as the source of

fast atoms. The primary beam impacted on the sample at 8.5 KeV with a beam flux equivalent to 1mA. The secondary ions produced were accelerated to 8 KeV from the source region and analysed in the first mass spectrometer (EB).

Full scans were performed using a scan speed of 10 s per decade at a resolution of 1500 over the mass range m/z 1050 to m/z 50. Samples, in chloroform, were analysed by dissolving 2 μl of sample in 2 μl of 3-amino-1,2-propanediol (Aldrich) which served as the FAB mass spectrometry (MS) matrix.

Accurate mass analyses were performed using data-system assisted ''peak matching'' at a resolution of 10 000. Glycerol (Aldrich) was used as the matrix and glycerol ions used for peak matching purposes. Two sets of 10 analyses were taken and these then averaged to give the ppm error relative to the chemical formula for the proposed structure of metabolite M2.

2.10. Nuclear magnetic resonance (NMR)

BPAU and purified M1, M2 or M3 were dissolved in DMSO. Scanning was carried out on a Bruker ARX 250 instrument. The scanning times varied with the sample quantity.

3. Results and discussion

3.1. Separation of BPAU and its metabolites by HPLC

HPLC graphs (Fig. 1B and D) clearly show that BPAU and its metabolites were well separated under the analytical conditions established in this study. The retention times of BPAU and its metabolites, were 5.5 min (M2), 7.5 min (M3), 9.4 min (M1) and 12.6 min (BPAU). Fig. 1A and B show the HPLC graphs of the BPAU standard and the serum sample from a rat 6 h after BPAU dosing. The recovery of BPAU spiked sample was $94.2 \pm 3.7\%$ ($n=5$). Therefore, this method is suitable for quantitative analysis of BPAU. Ammonium acetate significantly influenced the chromatographic performance of M2. In the absence of ammonium acetate in the mobile phase, the peak of M2 was significantly broadened and even disappeared, whereas M1, M3 and BPAU were not affected by ammonium acetate. Adjusting pH alone improved M2 HPLC performance but was not satisfactory. So the presence of the ammonium salt plays an important role in M2 separation. Therefore, ammonium acetate in the mobile phase was necessary for M2 analysis.

BPAU and its metabolites M1, M2 and M3 were stable in tissue, serum or urine extraction stored at 4°C for two weeks as checked by HPLC.

3.2. Characterisation of BPAU and its metabolites by MS and NMR spectra

3.2.1. BPAU

The mass spectrum of starting material, BPAU, is shown in Fig. 2. The molecular ion of BPAU is at m/z 255/257. The double peak resulted from isotopes of bromine (Br) which also served as a natural MS marker for its metabolites, M1, M2 and M3, see below. Two major fragment ions at m/z 79/81 and m/z 177 were observed. They represent the ions of Br and phenylacetylurea, respectively and indicate that the cleavage took place at the Br–C bond during ionisation.

The NMR spectrum of BPAU showed four protons on the benzene ring clearly displayed between δ 7.2 and δ 7.7 ppm. A single resonant peak of two protons on the acetyl carbon connected with aromatic ring was seen at δ 2.5 ppm. The remaining protons were not clearly identified because protons on the primary and secondary nitrogens are often broadened or invisible [11].

3.2.2. M1

The mass spectrum of M1 (Fig. 3) displayed a molecular ion at m/z 271/273 and three major fragment ions at m/z 79/81, 192 and 228/230, respectively. The molecular ion is 16 mass units more than its parent compound, BPAU. This suggests that an oxygen, [O], was added to BPAU to form M1. The fragment ion peaks at m/z 79/81 and m/z 192 indicate the cleavage at the Br-bond of M1 during ionisation. The ion peak at m/z 228/230 resulted from the cleavage between the secondary N and the carbon of urea, N–C(O). This indicates that the [O] was not added to the primary nitrogen and

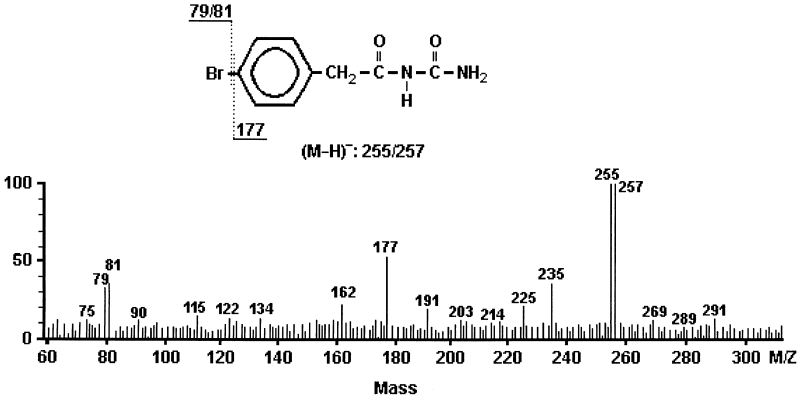

Fig. 2. Mass spectrum of BPAU.

implies that the [O] may be added to the secondary nitrogen because the hydroxylation of the secondary nitrogen weakened the bond between the secondary nitrogen and the urea carbon and caused cleavage during ionisation. This cleavage did not happen with the parent compound, BPAU (Fig. 2).

The NMR spectrum of M1 showed that a single broadened proton peak was observed at δ 11.8 ppm which represents the proton on =N–O–H. Two doublet proton peaks were observed between δ 7.3 ppm and δ 7.8 ppm which represent four protons on the aromatic ring of M1. This clearly indicates that no substitution took place on the aromatic ring of BPAU. A single peak of two protons on the acetyl

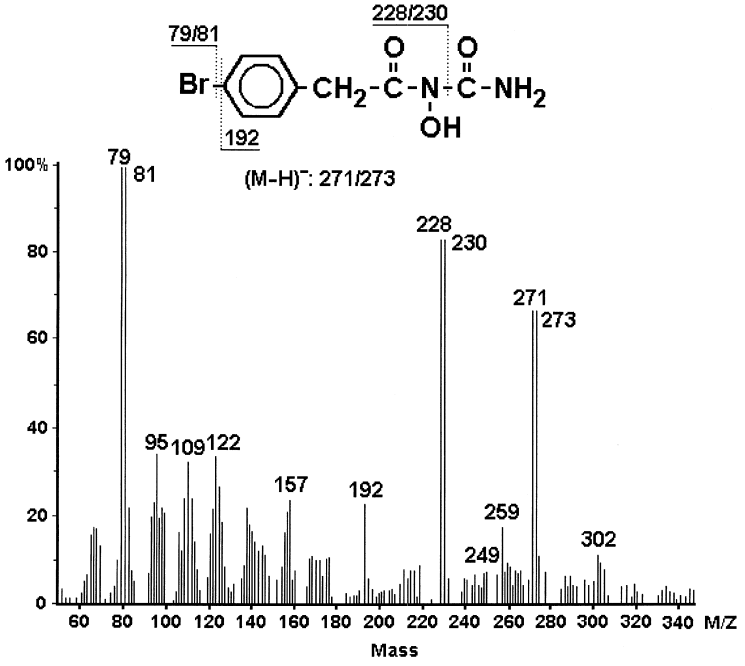

Fig. 3. Mass spectrum of M1.

carbon connected with aromatic ring was seen at δ 2.5 ppm. Thus the molecular structure of M1 was characterised.

According to the IUPAC nomenclature system of chemicals, M1 is named 3-hydroxy-5-(4-bromophenyl)-1,3-diazapentane-2,4-dione. Its common name is called *N′*-hydroxy-*p*-bromophenylacetylurea (HBPAU).

3.2.3. M2

The molecular ion peak of M2 was seen at m/z 254/256 which is one mass unit less than its parent compound, BPAU (Fig. 4). Its fragment ions were seen at m/z 79/81 and m/z 176 which indicate that the cleavage took place between Br and the rest of M2 during ionisation. The double ion peak indicates that the Br atom is still on the molecule of the metabolite. The mass spectrum of M2 did not provide enough information for working out its molecular structure and an accurate mass spectrum of M2 was carried out. The accurate MS indicated that M2 is composed of nine C, six H, three O, one N and one Br (Fig. 5). This shows that M2 is one oxygen more and one nitrogen and three hydrogens less than BPAU (nine C, nine H, two O, two N and one Br).

The NMR spectrum shows that two doublet proton

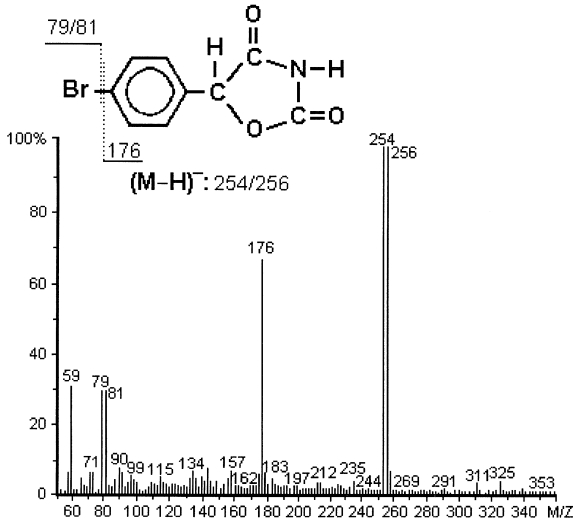

Fig. 4. Mass spectrum of M2.

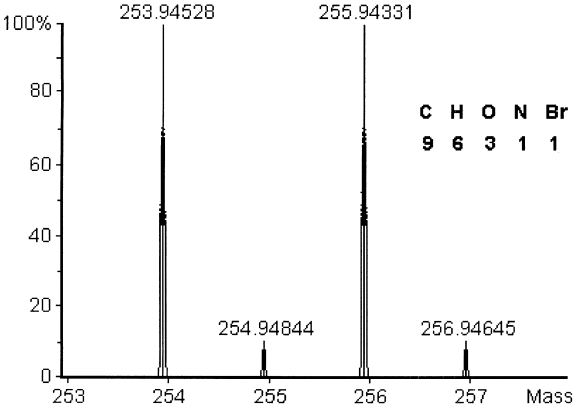

Fig. 5. Accurate mass spectrum of M2.

peaks were observed between δ 7.3 ppm and δ 7.8 ppm which indicate that the four protons on the aromatic ring were not replaced. A single proton peak was observed at δ 6.0 ppm which is likely to result from the resonance of the proton on the acetyl carbon connected with aromatic ring. Thus, chemically, a ring structure shown in Fig. 4 matches both the mass spectrum and the accurate mass spectrum. So the M2 molecular structure was characterised.

According to IUPAC nomenclature system, M2 is named 4-(4-bromophenyl)-3-oxapyrrolidine-2,5-dione.

3.2.4. M3

M3 has a molecular ion peak at m/z 269/271 (Fig. 6). It is two mass units less than M1 and 14 mass units more than BPAU. Three fragment ion peaks are seen at m/z 79/81, 175 and 191. The ion peaks of m/z 79/81 and 191 resulted from the cleavage between Br and the remainder of M3. The ion peak of 175 may represent that the $-CH_3$ group came off from the ion 191 during ionisation.

The NMR spectrum of M3 indicates that protons on the benzene ring remained unchanged. The resonant peaks of protons on $-CH_3$ was not identified because they overlapped with the resonant peaks of the protons on the DMSO (solvent). A peak of the protons on the acetyl carbon connected with aromatic ring was observed at δ 2.5 ppm. The protons on the primary nitrogen were not observed because the

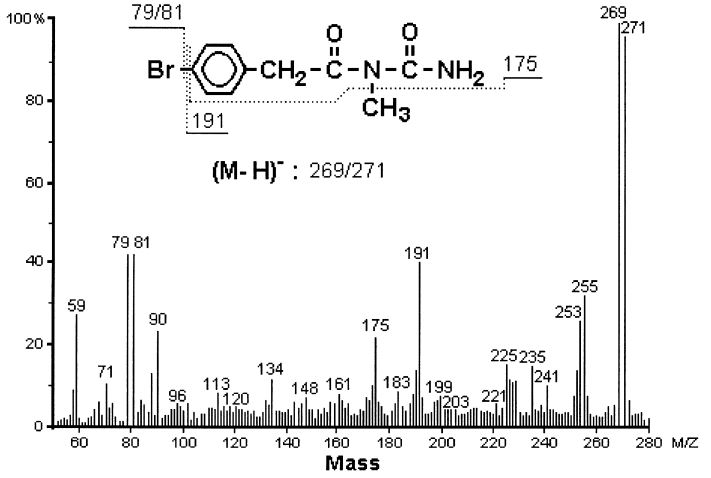

Fig. 6. Mass spectrum of M3.

resonance peak of protons on nitrogen is often broadened or invisible [11]. Chemically, therefore, it is suggested that methylation is likely on the secondary nitrogen of BPAU, although this will need further confirmation.

According to IUPAC nomenclature system, M3 is named 3-methyl-5-(4-bromophenyl)-1,3-diazapentane-2,4-dione. Its common name is *N′*-methyl-*p*-bromophenylacetylurea (MBPAU).

3.3. Metabolic pathways of BPAU

The metabolic pathways of BPAU are proposed as shown in Fig. 7. The enzymes involved in these

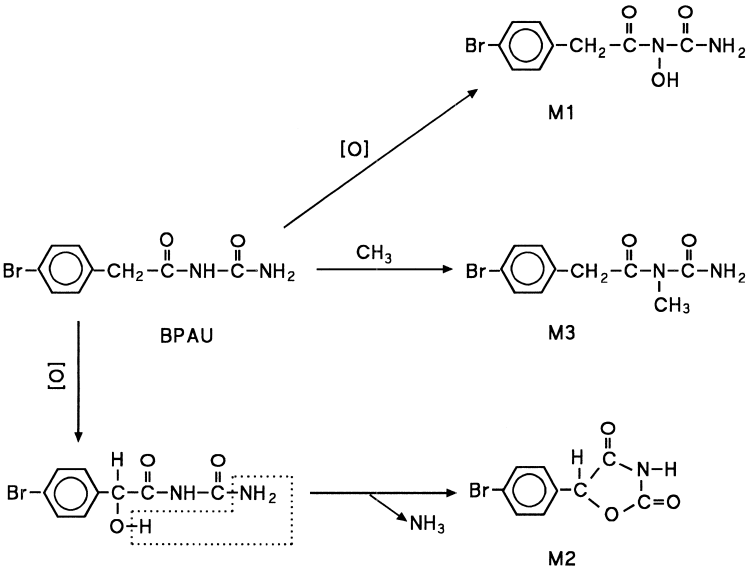

Fig. 7. Proposed metabolic pathways of BPAU.

pathways have not been studied yet. M1 was transformed from BPAU by hydroxylation on its secondary nitrogen. It has been demonstrated that N-oxidation of secondary or tertiary amines is catalysed by flavin-containing monooxygenase (FMO) [12–15]. So, FMO is likely to catalyse the hydroxylation of the secondary nitrogen on BPAU to form M1. At least two steps were involved in M2 formation. The initial step is the hydroxylation of the aliphatic carbon connecting with the aromatic ring. This reaction is usually catalysed by cytochrome P-450 enzyme system [15]. M3 is formed by the methylation of the secondary nitrogen on BPAU. Various specific and non-specific *N*-methyl transferases have been identified in mammals. These *N*-methyl transfereases are capable of methylating a variety of primary, secondary and tertiary exogenous and endogenous amines [15]. Further studies are needed to clarify the enzymes involved in BPAU metabolism.

4. Summary

This study provides for the first time a HPLC method for the isolation and purification of BPAU and its metabolites and characterised three metabolites of BPAU by MS and NMR techniques. The establishment of an analytical method for BPAU and its metabolites now makes it possible to further study its pharmacokinetics. Identification and characterisation of metabolites are the key steps in studies of drug or other xenobiotic metabolism. This study suggests that HPLC is a useful method to separate and purify metabolites that are crucial for further characterisation by MS and NMR techniques.

Acknowledgements

The authors wish to thank Dr. P.B. Farmer and Dr. C.-K. Lim (MRC Toxicology Unit, Leicester, UK), for their help and discussions on the characterisation of BPAU and metabolites and Dr. R.M. Ellam (University of Hertfordshire, Hatfield, UK) for his help in the nomenclature of BPAU metabolites. This study was supported by MRC Toxicology Unit, Leicester, UK.

References

[1] B. Cavanagh, F.C.-K. Chen, J. Neurosurg. Psychiatry 31 (1968) 471.
[2] B. Diezel, G. Quadbeck, Pharmakol. Exp. Pathol. 238 (1960) 534.
[3] J. Jakobsen, E.H. Lambert, G. Carlson, S. Brimijoin, Exp. Neurol. 75 (1982) 173.
[4] J.C. Troncoso, J.W. Griffin, D.L. Price, K.M. Hess-Kozlow, Lab. Invest. 46 (1982) 215.
[5] L.H. Fossom, R.B. Messing, S.B. Sparber, NeuroToxicology 6 (1985) 17–28.
[6] H. Nagata, S. Brimijoin, Brain Res. 385 (1986) 136–142.
[7] H. Nagata, S. Brimijoin, P. Low, J.D. Schmelzer, Brain Res. 422 (1987) 319–326.
[8] N. Oka, S. Brimijoin, Brain Res. 509 (1990) 107–110.
[9] B.S. Jortner, K. Dyer, A. Walton, M. Ehrich, NeuroToxicology 18 (1997) 161–168.
[10] C.-K. Chen, L.B. Cavanagh, Br. Exp. Pathol. 52 (1971) 315.
[11] W. Kemp, in: Organic Spectroscopy, 2nd ed., Macmillan, Hampshire, London, 1987, pp. 83–157.
[12] D.M. Ziegler, Drug Metab. Rev. 19 (1988) 1–32.
[13] M. Ziegler, Trends. Pharmacol. Sci. 11 (1990) 321–324.
[14] E. Tynes, E. Hodgson, Arch. Biochem. Biophys. 240 (1985) 77–93.
[15] I.G. Sipes, A.J. Gandolfi, in: M.O. Amdur, J. Doull, C.D. Klaassen (Eds.), Casarett and Doll's Toxicology – The Basic Science of Poisons, 4th ed., McGraw-Hill, New York, 1991, pp. 88–126.