Note

High-performance liquid chromatography with on-line radioactivity monitoring for metabolic studies of 2- and 4-[14C]aminobiphenyls

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Isomeric 2- and 4-aminobiphenyl are known to differ greatly in their toxicological properties towards experimental animals. 2-Aminobiphenyl (2-ABP) has little or no carcinogenic or mutagenic activity[1–3] in most species, whereas 4-aminobiphenyl (4-ABP) is a potent carcinogen in many species including man[4–6] and is highly mutagenic[1,7,8]. These aromatic amines occur in the environment and represent an occupational hazard to workers in the chemical industry[9,10]. Since metabolic conversion is a necessary step in the bioactivation of the carcinogenic amines and amides[11,12], comparative metabolism of these isomers with different species is of interest. Our laboratory has been involved in providing metabolic data on the isomeric aminobiphenyls[13–15]. To achieve this we have investigated various analytical methods for the amines and their metabolites. We now report on techniques based on reversed-phase high-performance liquid chromatography (HPLC) and radiometry for obtaining *in vitro* and *in vivo* metabolic profiles of two isomeric aminobiphenyls.

EXPERIMENTAL

Materials
Potential oxidative metabolites of 4-amino- and acetamidobiphenyl, radioactive compounds and liquid scintillation materials were obtained as previously described[7,14,16]. Corresponding amino and acetamido derivatives of 2-aminobiphenyl were similarly obtained as described elsewhere[15,17]. Reference standards for water-soluble conjugated metabolites were prepared as follows: potassium salts of 2-amino-3-hydroxybiphenyl and 4-amino-3-hydroxybiphenyl-O-sulphates were prepared from the corresponding parent amines by persulphate oxidation in alkaline solution[18]. The O-sulphates of acetamidophenols and the N-sulphates of the parent amines were prepared as their ammonium salts after reaction with chlorosulphonic acid in dry pyridine[19]. Arylamine N-glucuronides were prepared as their sodium salts by reacting the parent amines with sodium glucuronate according to the general method[18]. Radioactive O-glucuronides of the phenolic amines and amides were biosynthesised by incubating each aglycone with guinea pig liver microsomal fraction fortified with [14C]uridine diphosphate glucuronic acid and magnesium chloride[20,21].

High-performance liquid chromatography

The liquid chromatograph used consisted of the following: two Beckman Model 110A pumps interfaced with an Altex Model 420 microprocessor solvent gradient programmer, a Pye Unicam LC3 UV spectrophotometer, a Gilson dual pen chart recorder; analyses were performed on C_{18}-bonded Hypersil 5 ODS columns (25.0 cm × 4.6 mm I.D. HPLC Technology, Wilmslow, U.K.) protected by a pre-column (50 × 5 mm) packed with Co-Pel 10 ODS (Whatman, Maidstone, U.K.). All HPLC solvents were filtered through Sartalon (Nylon 66) (Sartorius Instruments, Surrey, U.K.). Tetrabutylammonium hydroxide (TBAH, 0.5 M solution) was obtained from Fisons, U.K. Radioactivity of compounds in the HPLC eluate was monitored on-line using a Flo-One Model HP radioactive detector (Radiomatic Instruments & Chemical Co., U.S.A.) equipped with a 500-μl liquid cell. The scintillation fluid was delivered by an in-built peristaltic pump at a predetermined flow-rate of 3-ml/min which was mixed with a fraction of the HPLC eluate (1 ml/min) in a mixing chamber before reaching the flow cell for counting. The counting data was processed using a computerized data system (CP/M Micromate).

Sample preparation

Standard solutions of reference compounds in methanol (30–50 μl) were injected into the high-performance liquid chromatograph. An aqueous mixture containing all the reference compounds of each amine was prepared in a screw-cap Sovirel tube by pooling aliquots of the standard solutions and diluting with water. This solution was subjected to solvent extraction with diethyl ether at pH 6.0 to remove soluble compounds. The pooled diethyl ether phase was evaporated under nitrogen and the residue redissolved in methanol for HPLC analysis. Extraction of water-soluble conjugates was achieved by passing the remaining aqueous phase, after diethyl ether extraction, through a C_{18}-bonded Sep-Pak cartridge and subsequent elution of the adsorbed compounds with methanol. The methanol was concentrated on a vortex evaporator and the residue was redissolved in methanol prior to HPLC analysis. Several programmes both in the isocratic and gradient modes were tested in order to find the best separating conditions for the mixtures.

Aliquots of urine samples obtained from rats dosed with ^{14}C-labelled 2- or 4-ABP were analysed as above using the best chromatographic conditions found.

RESULTS AND DISCUSSION

Attempts to separate all the diethyl ether-soluble derivatives of either isomer in the isocratic mode at any one composition of methanol–water or acetonitrile–water were unsuccessful. At low acetonitrile or methanol concentration in the mobile phase the phenolic compounds and the hydroxylamines were eluted well at short retention times whereas the amine and other less polar derivatives were poorly eluted at very long retention times. The use of different gradient systems enabled separation of all the diethyl ether-extractable compounds derived from each amine. The best HPLC conditions established are shown in Table I. Although the use of acetonitrile–buffer or acetonitrile–water as mobile phase was effective in resolving all the diethyl ether-soluble derivatives, 4-acetamido-N-hydroxybiphenyl was strongly retained in the reversed-phase column. This observation is in accordance with earlier

TABLE I

HPLC CHARACTERISTICS OF DIETHYL ETHER-SOLUBLE DERIVATIVES OF 2-ABP AND 4-ABP

P_1: Flow-rate, 1.5 ml/min; time, 0 min, pump B = 35%; time, 2 min, pump B = 35–65% in 8 min; time, 15 min, pump B = 65–80% in 3 min; time, 21 min, pump B = 80–35% in 3 min. P_2: Flow-rate, 1.5 ml/min; time, 0 min, pump B = 30%; time 2 min, pump B = 30%; time 2 min, pump B = 30–40% in 8 min; time 11 min, pump B = 40–55% in 8 min; time, 20 min, pump B = 55–80% in 3 min time, 28 min, pump B = 80–30% in 2 min. ND = not determined.

Compounds	t_R (min) P_1*	Compounds	t_R (min) P_2*	P_2**
2-Aminobiphenyl (2-ABP)	12.0	4-Aminobiphenyl (4-ABP)	18.8	18.6
2-Amino-3-hydroxybiphenyl (2-ABP-3-OH)	9.0	4-Amino-4'-hydroxybiphenyl (4-ABP-4'-OH)	7.6	7.2
2-Amino-4'-hydroxybiphenyl (2-ABP-4'-OH)	7.2	4-Amino-3-hydroxybiphenyl	13.4	12.0
2-Amino-5-hydroxybiphenyl (2-ABP-5-OH)	6.0	4-Amino-2'-hydroxybiphenyl (4-ABP-2'-OH)	10.2	9.6
2-Hydroxylaminobiphenyl (2-NH-OH)	9.8	4-Hydroxylaminobiphenyl	15.2	14.8
2-Nitrosobiphenyl (2-NOBP)	15.0	4-Nitrosobiphenyl	26.2	26.2
2-Nitrobiphenyl (2-NBP)	13.6	4-Nitrobiphenyl	25.4	25.8
2-Acetamidobiphenyl	7.2	4-Acetamidobiphenyl (4-AABP)	16.2	16.0
2-Acetamido-3-hydroxybiphenyl	4.6	4-Acetamido-4'-hydroxybiphenyl (4-AABP-4'-OH)	6.6	6.6
2-Acetamido-4'-hydroxybiphenyl	3.8	4-Acetamido-3-hydroxybiphenyl	13.4	13.0
2-Acetamido-5-hydroxybiphenyl	2.8	4-Acetamido-2'-hydroxybiphenyl (4-AABP-2'-OH)	9.0	9.0
2-Acetamido-N-hydroxybiphenyl	6.2	4-Acetamido-N-hydroxybiphenyl	ND	13.8
N-(2-Biphenylyl)glycolamide	6.6	N-(4-Biphenylyl)glycolamide	11.4	11.6
N-(2-Biphenylyl)oxamic acid	1.6	N-(4-Biphenylyl)oxamic acid	2.5	3.8
N-Formyl-2-aminobiphenyl	8.5	4,4'-Bis-azoxybiphenyl	27.4	27.4
2,2'-Bis-azoxybiphenyl	20.2	4,4'-Bis-azobiphenyl	28.2	28.2
2,2'-Bis-azobiphenyl	22.0	N-Formyl-4-aminobiphenyl	16.0	15.8

* Water (pump A)–acetonitrile (pump B).
** 0.1 M acetate buffer plus acetohydroxamic acid, 0.5% (w/v), pH 4.4 (pump B).

workers[22] who reported on the HPLC characteristics of other arylhydroxamic acids. The use of a buffer system containing acetohydroxamic acid to presaturate the adsorptive sites of the reversed-phase material as suggested by the above workers was found successful for the elution of the 4-acetamido-N-hydroxybiphenyl acid together with the other compounds. Furthermore this mobile phase provided better resolution between the arylhydroxylamine and 4-amino-3-hydroxybiphenyl than the systems without acetohydroxamic acid. Reversed-phase HPLC with gradient elution has previously been used for separation of 4-ABP and its hydroxylated products in microsomal suspensions[22]. However this earlier method did not include acetylated derivatives of the amine. The present improved method enables complete separation of both amino and acetamido compounds in a single chromatographic run. Fig. 1 shows representative chromatograms of diethyl ether-extractable metabolites from urine of rat dosed with the radiolabelled amines.

The HPLC characteristics of sulphate and glucuronide conjugates of the amines and their derivatives are summarised in Table II. These compounds were found to be poorly retained on reversed-phase columns when a mobile phase of acetonitrile–buffer or acetonitrile–water was used for elution at a number of com
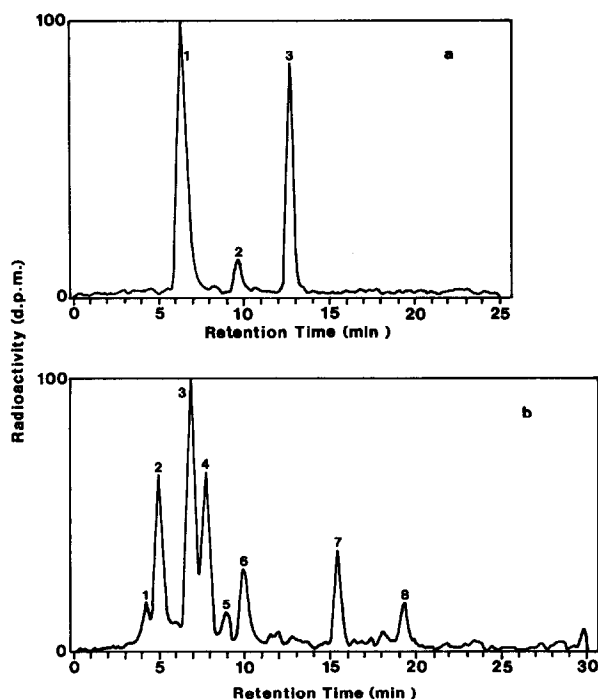
Fig. 1. Representative HPLC radiochromatograms of diethyl ether-soluble metabolites in urine of rats dosed with (a) ^{14}C-labelled 2-ABP and (b) ^{14}C-labelled 4-ABP. Peaks in a: 1 = 2-ABP-5-OH; 2 = 2-ABP-3-OH; 3 = 2-ABP. Peaks in b: 1 = N-(4-biphenylyl)oxamic acid; 2 = unknown; 3 = 4-AABP-4'-OH; 4 = 4-ABP-4'-OH; 5 = 4-AABP-2'-OH; 6 = 4-ABP-2'-OH; 7 = 4-AABP; 8 = 4-ABP. Abbreviations as in Table I.

positions examined. This is because such conjugates are generally ionised in aqueous solutions above pH 3.0. Although such ionisation can be suppressed by using a mobile phase at pH < 3.0, e.g. a system containing acetic acid, to improve the retention properties of conjugates, low pH values can cause deterioration of reversed-phase column materials. Furthermore, the use of acidic media causes breakdown of acid-labile compounds such as amine N-glucuronides[24,25].

The use of a mobile phase containing an ion-pairing reagent, TBAH, buffered at pH 7.0 increased the retention times of all the conjugates. Under isocratic conditions the glucuronides eluted first at short retention times whereas the sulphates had relatively very long retention times. At this pH the acid-labile N-glucuronides were stable during the chromatographic run. The best conditions for separation of mixtures of sulphates and glucuronides were therefore obtained using gradient techniques. Representative chromatograms showing separation of conjugates in rat urine after administration of the amines are shown in Fig. 2. These observations are in accordance with reports of the HPLC separation of conjugates of other compounds[26,27].

Previous studies on the metabolism of 2-ABP[17] and 4-ABP[27] involved the use of paper or thin-layer chromatography for the characterisation of glucuronides and sulphates. Such techniques are slow, laborious and inefficient in providing detailed

TABLE II

HPLC CHARACTERISTICS OF SULPHATES AND GLUCURONIDES OF 2- AND 4-AMINOBIPHENYL DERIVATIVES

P_3: Flow-rate, 1.5 ml/min; time, 0 min, time, 2 min, pump B = 30%, pump B = 30–60% in 20 min; time 25 min, pump B = 60–80% in 2 min, time, 28 min, pump B = 80–30% in 2 min.

Compounds	t_R (min) P_3^*	Compounds	t_R (min) P_3^*
Potassium 2-aminobiphenylyl sulphamate (2-AB-NS)	9.0	Potassium 4-aminobiphenylyl sulphamate	22.0
Potassium 2-amino-3-biphenylyl sulphate (2-ABP-3-OS)	12.0	Potassium 4-amino-3-biphenylyl sulphate (4-ABP-3-OS)	23.8
Potassium 2-amino-4'-biphenylyl sulphate	8.2	Potassium 4-acetamido-4'-biphenylyl sulphate (4-AABP-4'-OS)	17.0
Potassium 2-amino-5-biphenylyl sulphate (2-ABP-5-OS)	6.6	Potassium 4-acetamido-3-biphenylyl sulphate	24.8
Potassium 2-acetamido-3-biphenylyl sulphate	5.8	Potassium 4-acetamido-2'-biphenylyl sulphate	18.4
Potassium 2-acetamido-4'-biphenylyl sulphate	4.5	Sodium 4-aminobiphenylyl N-glucuronide	16.4
Potassium 2-amino-5-biphenylyl sulphate	3.2	4-Amino-4'-biphenylyl glucuronide (4-ABP-4'-OG)	7.5
Sodium 2-aminobiphenyl N-glucuronide (2-ABP-NG)	2.4	4-Amino-3-biphenylyl glucuronide	13.4
2-Amino-3-biphenylyl glucuronide (2-ABP-3-OG)	5.6	4-Acetamido-4'-biphenylyl glucuronide (4-AABP-4'-OG)	6.0
2-Amino-4'-biphenylyl glucuronide	3.5	4-Acetamido-3-biphenylyl glucuronide	14.2
2-Amino-5-biphenylyl glucuronide (2-ABP-5-OG)	3.9	4-Acetamido-2'-biphenylyl glucuronide (4-AABP-2'-OG)	9.4
2-Acetamido-3-biphenylyl glucuronide	4.2	4-Acetamidobiphenyl-N-hydroxy O-glucuronide	15.0
2-Acetamido-4'-biphenylyl glucuronide	3.0	(4-AABP-N-OG)	
2-Acetamido-5-biphenylyl glucuronide	3.4		

* 0.02 M phosphate buffer containing 0.005 M TBAH, final pH = 7 (pump A), acetonitrile–0.02 M phosphate buffer containing 0.005 M TBAH (70:30, v/v), pH = 7.0 (pump B).

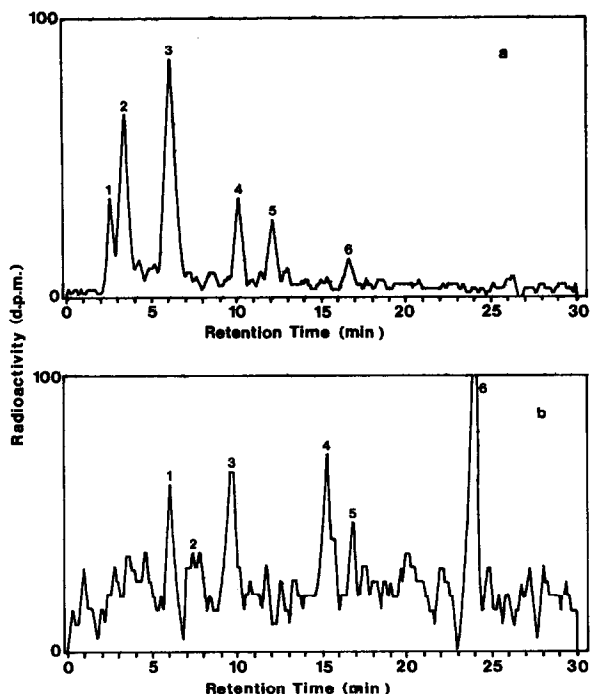Fig. 2. Representative HPLC radiochromatograms of water-soluble metabolites in urine of rats dosed with (a) ^{14}C-labelled 2-ABP; (b) ^{14}C-labelled 4-ABP. Peaks in a: 1 = 2-ABP-NG; 2 = 2-ABP-5-OG; 3 = 2-ABP-5-OS; 4 = 2-ABP-NS; 5 = 2-ABP-3-OS; 6 = unknown. Peaks in b: 1 = 4-AABP-4'-OG; 2 = 4-ABP-4'-OG; 3 = 4-AABP-2-OG; 4 = 4-AABP-N-OG; 5 = 4-AABP-4'-OS; 6 = 4-ABP-3-OS. Abbreviations as in Table II.

metabolic profiles of compounds. The present methods were found to be rapid, efficient and convenient for studying both phase I and phase II metabolites[28] without prior hydrolytic procedures.

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