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Solid phase extraction and HPLC determination of 9-benzyladenine and isomeric 9-(nitrobenzyl)adenines and their metabolic N^1-oxides present in microsomal incubates

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Abstract

9-Benzyladenine, 9-(2-nitrobenzyl)adenine, 9-(3-nitrobenzyl)adenine and 9-(4-nitrobenzyl)adenine were metabolized to 9-benzyladenine-N^1-oxide, 9-(2-nitrobenzyl)adenine-N^1-oxide, 9-(3-nitrobenzyl)adenine-N^1-oxide and 9-(4-nitrobenzyl)adenine-N^1-oxide, respectively, by animal hepatic microsomes. For the quantitative determination of the substrates and metabolites present in microsomal incubates, an off-line solid phase extraction procedure, using columns packed with C18 silica bonded phase, was developed. The extraction recovery for these 9-alkyladenines and their N^1-oxides was in the range of 92–101%. A reversed-phase HPLC method was established with an ODS column at a column temperature of 50°C. The mobile phase consisted of H_2O-MeOH-diethylamine (65:35:0.5, v/v/v), pH 6.8. The above analytes were monitored at 233 nm and the retention times of all analytes were within 6–14 min. The within-day coefficients of variation (CV) for the determinations were in an acceptable range. The biotransformation of BA and NBAs to N^1-oxides by hamster microsomes was determined under the experimental conditions employed.

Keywords: Solid phase extraction; Reversed-phase chromatography; Hepatic microsomal oxidation; Metabolism; 9-Alkyladenine metabolism; 9-Alkyladenine-N^1-oxides

1. Introduction

The biological N^1-oxidation of adenine and some of its 9-substituted derivatives and possible metabolic controlling factors have been previously investigated and discussed [1–5]. As a continuation of the previous research and in order to establish structure-metabolism relationships, the metabolic N^1-oxidation of 9-benzyladenine (BA) and isomeric 9-(nitrobenzyl)adenines (NBAs) by hamster hepatic microsomes was studied [6]. The solid phase extraction (SPE) method and the reversed-phase HPLC procedure employed in these studies are presented in this report.

SPE methods have been widely applied for the purification, extraction and isolation of compounds from various sample sources. The methodology, application, sorbent materials as well as other related aspects of SPE methods have

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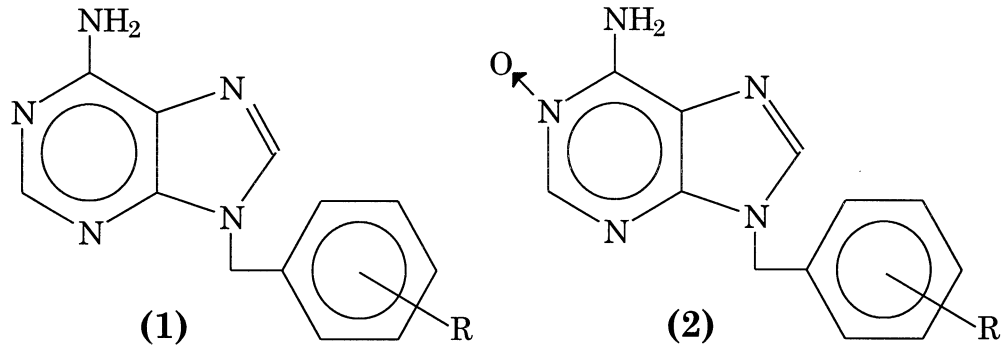

Substrate			Metabolite
R=H:	9-benzyladenine	(BA)	9-benzyladenine-N^1-oxide (BANO)
R=2-nitro:	9-(2-nitrobenzyl)adenine	(2NBA)	9-(2-nitrobenzyl)adenine-N^1-oxide (2NBANO)
R=3-nitro:	9-(3-nitrobenzyl)adenine	(3NBA)	9-(3-nitrobenzyl)adenine-N^1-oxide (3NBANO)
R=4-nitro:	9-(4-nitrobenzyl)adenine	(4NBA)	9-(4-nitrobenzyl)adenine-N^1-oxide (4NBANO)

Fig. 1. Structures and abbreviations of 9-alkyladenines and their N^1-oxides studied.

recently been extensively reviewed [7,8]. To extract BA, NBAs and their N^1-oxides, i.e., 9-benzyladenine-N^1-oxide (BANO) and isomeric 9-(nitrobenzyl)adenine-N^1-oxides (NBANOs), from microsomal incubates, a convenient SPE procedure with high extraction recovery was developed and evaluated. Although a cationic exchange HPLC method, which was suitable for the determination of adenine, 9-methyladenine, BA and certain other adenine 9-substituted derivatives as well as their potential metabolic N^1-oxides [1–3], was previously established and used in our laboratory, the present reversed-phase HPLC method was superior for rapid and sensitive detection of BA, NBAs and their N^1-oxides.

2. Materials and methods

2.1. Chemicals

Fig. 1 showed the structures and abbreviations of 9-alkyladenines and their N^1-oxides studied. They were synthesized from adenine and will be described in detail elsewhere. Glucose-6-phosphate (G-6-P) and nicotinamide adenine dinucleotide phosphate (NADP) were obtained from Sigma Chemical (Dorset, UK). Glucose-6-phosphate-dehydrogenase suspension (grade II) was purchased from Boehringer Mannheim (Sussex, UK). *N*,*N*-Diethylamine, *N*,*N*-dimethylformamide (DMF) (HPLC grade) were the products of Aldrich Chemical (Dorset, UK). Other chemicals and solvents used were either analytical reagent or HPLC grade and were supplied by Aldrich Chemical (Dorset, UK), Lancaster Synthesis (Lancashire, UK), or other sources.

2.2. Apparatus

The HPLC apparatus consisted of a M-45 Solvent Delivery System (Waters, Milford, USA), a SpectroMonitor 3100 Variable Wavelength Detector (LDC/Milton Roy, Riviera Beach, USA) and a CI-4100 Computing Integrator (LDC/Milton Roy, Clare, Ireland). A Spherisorb 5 ODS (2) column (25×0.46 cm particle size 5 μm; Phenomenex, Cheshire, UK) was used for HPLC

method. The SPE procedure utilized ISOLUTE C18 columns (non-end-capped, Lot No. 4131316DB) with 500 mg of sorbent mass and 6 ml of reservoir volume (Jones Chromatography, Mid Glamorgan, UK). One laboratory-made SPE sample processing station was used with 40-column capacity. An UVIKON 860 Spectrophotometer was produced by Kontron Instruments (Zürich, Switzerland).

2.3. Chromatographic conditions

The HPLC column temperature was kept at 50°C using a temperature controlled water bath. The isocratic mobile phase consisted of H_2O-MeOH-diethylamine (65:35:0.5, v/v/v) adjusted to pH 6.8 with H_3PO_4 (80% aq. v/v). For the simultaneous determination of BA and BANO, 4NBANO (10 nmol in 100 μl MeOH) was added to the samples as internal standard (IS), and BA (100 nmol in 100 μl MeOH) was used as the IS for the simultaneous determination of NBAs and NBANOs. The extracted samples were injected into a 20 μl valve loop and chromatographed with the mobile phase at 1.0 ml min^{-1} flow rate for 16 min. All of the samples were monitored at 233 nm at 0.05 a.u.f.s. The chromatograms were recorded and the peak heights were determined with an integrator. The amount of BA, BANO, NBAs and NBANOs in the incubates was determined from the respective calibration curves.

2.4. Calibration curves

For those standard samples, which were used for the establishment of calibration curves and the quantitation of analytes present in incubates, a simplified procedure was adopted without the use of inactivated animal microsomes. Known amounts of analytes were dissolved in DMSO (100 μl); IS solution (100 μl) and trichloroacetic acid solution (200 μl, 25%, w/v) were added and these standards were directly diluted with phosphate buffer (3 ml, 0.1 M, pH 7.4). The standards were applied to SPE columns for extraction (see below), chromatographed and the peak ratios of analytes to IS were obtained with an integrator. The calibration curves were constructed using peak height ratios against known concentrations of analytes. Additional experiments were carried out in the presence of inactivated microsomes (see later).

2.5. Incubations

Male Syrian hamsters (80–120 g) were supplied by King's College London animal facilities. The preparation of hamster hepatic microsomes and the incubations were carried out according to the standard procedures described by Altuntas and Gorrod [9]. Substrates were dissolved separately in DMF (10 μmol ml^{-1}) and 10 μl added to the hamster hepatic microsomal incubation system in a total volume of 1.5 ml. the incubations were carried out for 30 min at 37°C in a shaking incubator.

After incubation, the incubates were treated with trichloracetic acid (200 μl, 25% w/v in H_2O) to stop metabolism and precipitate proteins. IS solution (100 μl) was added and the incubates centrifuged at 3500 rpm for 10 min to remove proteins. The supernatants were collected, diluted with H_2O (1.5 ml) and applied to SPE columns for extraction (see below).

2.6. Sample extraction using the SPE procedure

The SPE columns were installed on the processing station, wetted with MeOH (2 ml) and conditioned with phosphate buffer (4 ml, 0.1 M, pH 7.4). The treated incubates and the standard samples were applied to the columns; interfering substances were washed away with phosphate buffer (4 ml, 0.1 M, pH 7.4) containing 15% MeOH. For desalination of columns, H_2O (2 ml) was used and any water remaining was removed by blowing air through the SPE columns for 1 min with a small air pump. Finally, the analytes were desorbed and eluted from the columns with MeOH (3 ml). The eluents were collected and evaporated using a Vortex-Evaporator (HAAK-Buchler Instrument, Saddle Brook, USA) under reduced pressure. The residues were dissolved in MeOH (300 μl) for HPLC analysis. During the whole the solutions were allowed to flow through the SPE column under gravity, unless the resistance of the

column blocked the flow. Under that situation, a syringe was connected to the column with an adapter to force the solution slowly through the column.

The SPE columns were reused up to 30 times without any apparent decrease of extraction capacity. However, the columns were regenerated after every 10 applications to remove any suspected hydrophobic contaminants. The regeneration was carried out by applying to the SPE columns MeOH (5 ml) followed by MeOH-methylene chloride (50:50 v/v, 10 ml) and again MeOH (5 ml).

2.7. Sample extraction using liquid/liquid procedures

For comparative purposes, standard solutions of 9-alkyladenines of interest were subjected to extraction by organic solvents. The samples (100 nmol analytes per 100 μl MeOH) in phosphate buffer (4 ml, 0.1 M, pH 7.4) were adjusted to pH 11 with NaOH; treated with either 4 ml of ethyl acetate, chloroform, methylene chloride or diethyl ether on a rocking device for 15 min and centrifuged. The organic phase was collected and evaporated under a stream of nitrogen. The residues were dissolved in MeOH (300 μl) and the peak heights established by HPLC compared to the non-extracted standards.

3. Results and discussion

3.1. Chromatography

Fig. 2 shows typical chromatograms and retention times of synthetic BA, BANO, NBAs and NBANOs. The results show that good separation and acceptable peak shapes of all analytes were achieved at 50°C HPLC column temperature under the chromatographic conditions employed. The compounds of interest derived from a single substrate can be separated in a single run.

The HPLC techniques used in the analysis of purine compounds including related nucleobases have been reviewed by Grune et al. [10]. It seems that reversed-phase HPLC, either in isocratic or gradient mode, has been commonly used for diverse purposes such as determining or separating purine nucleobases, ribonucleosides and ribonucleotides, although ion-pair reversed-phase HPLC was also useful in some cases to separate the bases from other nucleosides or nucleotides. A previously developed cationic exchange HPLC procedure in our laboratory was proved to be effective for the separation and determination of adenine, some 9-alkyladenines (such as 9-benzyladenine, 9-methyladenine, AMP and ATP etc.) and their potential N^1-oxide or N^6-hydroxylamine metabolites, with different mobile phases based on $NH_4H_2PO_4$-H_2O-MeOH or $NH_4H_2PO_4$-H_2O-acetonitrile systems [1–3].

However, the presently described reversed-phase HPLC method was shown to be more suitable for the rapid, convenient and simultaneous analysis of BA and BANO or NBAs and NBANOs present in microsomal incubation mixtures. For the present HPLC method, one important condition was to maintain the column temperature at 50°C. When the column temperature was ambient, BA and NBAs showed good peak shape while their N^1-oxides showed serious tailing. It was not difficult to obtain good peak

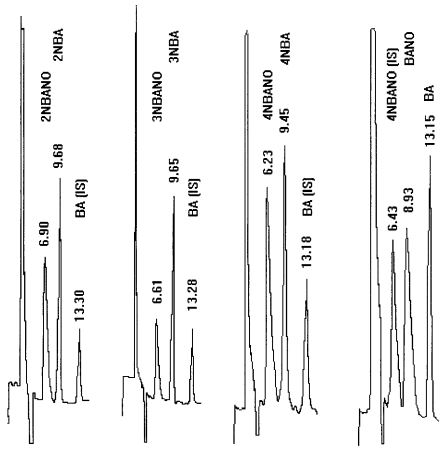

Fig. 2. Typical chromatograms of BA, BANO, NBAs and NBANOs on a Spherisorb 5 ODS (2) column (25 × 0.46 cm i.d.; particle size 5 μm) with column temperature 50°C. The mobile phase was H_2O-MeOH-diethylamine (65:35:0.5 v/v/v, adjusted to pH 6.8 with 80% H_3PO_4). Flow rate: ml min^{-1}; monitoring wavelength: 233 nm.

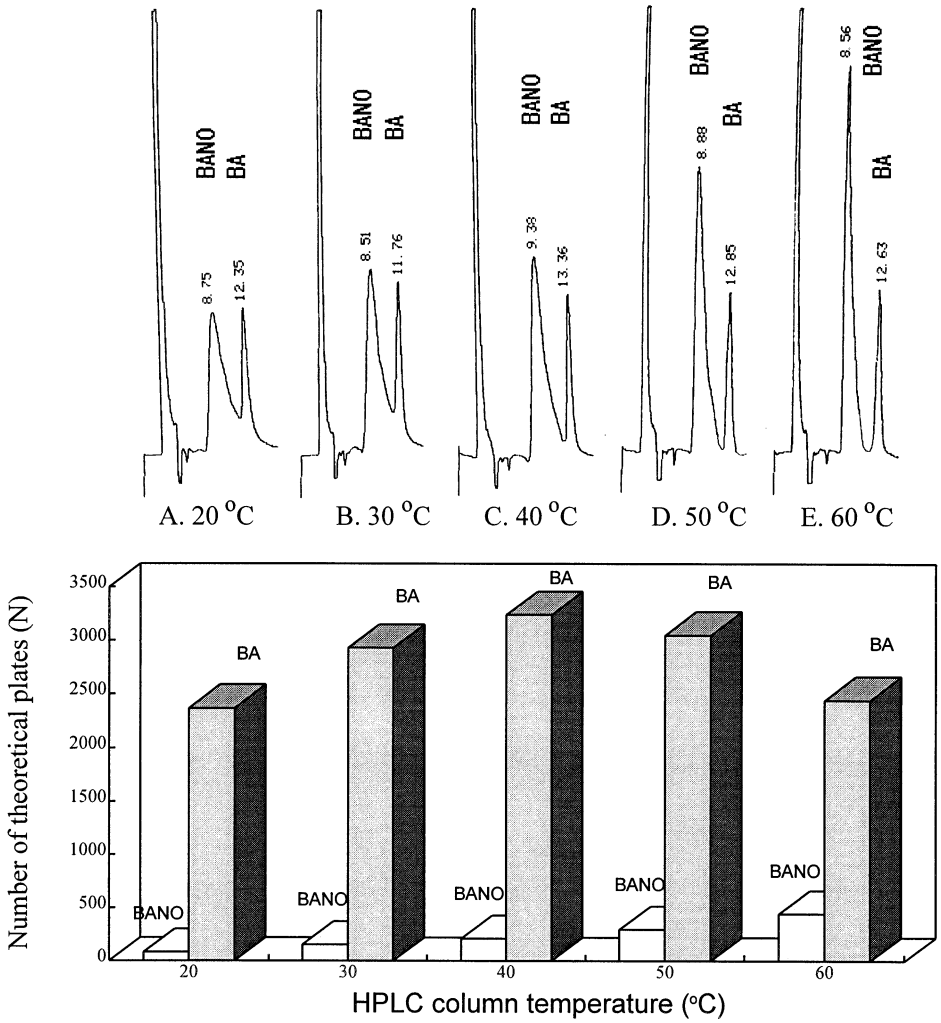

Fig. 3. Comparison of HPLC chromatograms of BA and BANO and theoretical plate numbers of the column for BA and BANO at different column temperatures. The HPLC conditions were: Spherisorb 5 ODS (2) column (25 × 0.46 cm i.d.; particle size 5 μm) with different column temperatures as indicted; flow rate: 1.0 ml min^{-1}; monitoring wavelength: 233 nm. The mobile phase contained diethylamine (0.5% v/v) and MeOH-H_2O (their ratios were: (A) 45:55; (B) 42:58; (C) 38:62; (D) 35:65; (E) 32:68 (v/v)), adjusted to pH 6.8 with 80% H_3PO_4.

shapes for BA and NBAs with an ODS column at room temperature, but it was very difficult to obtain satisfactory peak shapes for BANO and NBANOs under these conditions, even though several kinds of buffer systems and different pH values of mobile phase were tested. Increasing the column temperature is often an effective way to improve the peak shape of analytes. In the present case, this also greatly improved the peak shape of BANO and NBANOs, as indicated in Fig. 3. When the column was at room temperature (20°C), BANO showed serious tailing and the number of theoretical plates was only 82 while BA showed normal peak shape. When the
ture was raised to 50°C, the number of theoretical plates for BANO was increased three times and

BANO could be fully separated from BA. It was also possible to obtain a much higher theoretical plate number for BANO by using a higher column temperature, i.e., 60°C, but this temperature would markedly shorten the column life.

The introduction of a N-oxide group at the 1-position of the substrates makes the 9-alkyladenine N^1-oxide metabolites have quite different physico-chemical properties compared to their substrates, such as lower solubility in organic solvents and higher polarity. Higher column temperatures can decrease molecular interactions with the C18 sorbent and increase the solubility of the N-oxides in the mobile phase. This may be the mechanism of peak shape improvement of the N^1-oxides at higher column temperature.

The characteristics of the UV spectra of BA, BANO, NBAs and NBANOs in the mobile phase solution are shown in Table 1. BANO and NBANOs have maximum UV absorbance at 233 nm, while BA and NBAs showed low UV absorbance near the minimum at this wavelength (2.4–5.2 times lower than that at maximum absorbance wavelengths). This is an advantage of the present HPLC method as it is possible to determine not only the metabolite (N^1-oxide) but also the corresponding substrate in a single injection. As the substrates are always present at a much higher concentration than the N^1-oxides in the microsomal incubates, the concentration allowed the determination of the substrates at 233 nm even though they had low absorption at this wavelength. If greater sensitivity were required to assay the substrates in the absence of any N-oxide, then a detection wavelength of λ_{max} 2 was more suitable as monitoring wavelength as both the sensitivity and selectivity are higher.

Table 1
The UV spectral characterisitcs of BA, BANO, NBAs and NBANOs in the HPLC mobile phase

Compounds	λ_{max} 1 (nm)	λ_{max} 2 (nm)	Absorbance ratio	
			λ_{233nm}/ λ_{max} 1	λ_{max} 1/ λ_{max} 2
BA	261		0.28	
2NBA	261		0.46	
3NBA	262		0.35	
4NBA	263		0.36	
BANO	233	262		5.21
2NBANO	233	261		2.18
3NBANO	233	263		2.91
4NBANO	233	265		2.41

3.2. Sample preparation with SPE procedure

To evaluate the reproducibility and accuracy of the analytical method developed, the within-day coefficients of variation (CV) were determined for all analytes using the described SPE and HPLC procedures. In the concentration range 20–60 nmol per 1.5 ml sample for BANO and NBANOs, the CVs were in the acceptable range of 1.4–7.8% (Table 2). These results confirm that the method was reproducible and accurate.

SPE methods with versatile silica-bonded sorbents are useful techniques for the extraction of diverse analytes from various sample sources. Some applications of SPE have been reviewed, such as the extraction of pollutants from environmental materials [11], analysis of foods [12], trace enrichment of pesticides in water [13], extraction of some small drugs from plasma [14] or drugs and their metabolites from urine [15].

One of the outstanding advantages of SPE methods is that a high extraction recovery can usually be obtained for many compounds with a suitable sorbent and operating procedure, even under situations when other traditional extraction techniques, such as liquid extraction, may not be suitable. This was the case in our experiments. With the developed SPE procedure, very high extraction recoveries over 92% were obtained for all BA, NBAs, BANO and NBANOs (Table 2 and Table 3). Although some solvents examined could also reach a satisfactory extraction recovery for BA and NBAs, none of them were satisfactory for the extraction of NBANOs (Table 3). It is quite possible that the introduction of a N-oxide group into the NBAs greatly decreases the lipophilicity of these compounds and their solubility in organic solvents, so that it is not suitable to extract these N-oxides from aqueous samples with organic solvents. the results indicate that the SPE method established was ideal for this purpose.

Table 2
The within-day CVs of determination and the extraction recovery for the analytes by the SPE method and reversed HPLC procedure[a]

Analytes	Within-day CV (extraction recovery mean ± S.D. %)			
	20[b]	60[b]	100[b]	600[b]
BANO	6.7 (101 ± 6.7)	7.2 (96 ± 7.2)		
2NBANO	7.8 (93 ± 3.5)	3.8 (98 ± 3.7)		
3NBANO	1.7 (92 ± 1.6)	2.6 (94 ± 2.5)		
4NBANO	4.7 (100 ± 4.4)	5.8 (99 ± 5.5)		
BA			6.7 (103 ± 6.7)	1.4 (101 ± 1.4)
2NBA			2.4 (97 ± 2.2)	2.2 (96 ± 2.0)
3NBA			5.7 (98 ± 5.5)	3.2 (95 ± 3.0)
4NBA			3.5 (95 ± 3.2)	1.5 (100 ± 1.4)

[a] $n = 10$.
[b] Added amount of analytes (nmol per sample).

The extraction recovery of SPE was affected by several factors at different stages of the operation, such as the pH and ion concentration of solutions, proportion of organic solvent in the solutions, the speed of solutions passing through column and the selection of sorbent. The optimum SPE procedure could only be obtained after careful consideration and comparison of these factors. These aspects and the strategy to develop a SPE procedure were discussed in detail in a review prepared by the International Union of Pure and Applied Chemistry (IUPAC) [7]. To optimize the SPE procedure for BA, BANO, NBAs and NBANOs, some important factors, which might affect the extraction recovery of these analytes, were investigated using BA and BANO as models.

Table 3
Comparison of extraction recovery (%)

Analytes	Extraction methods				
	SPE	A	B	C	D
BANO	96	21	80	27	7
2NBANO	98	14	11	4	ND
3NBANO	92	36	10	10	2
4NBANO	99	59	66	51	ND
BA	101	96	99	99	77
2NBA	97	98	99	98	66
3NBA	98	77	91	84	42
4NBA	100	103	94	87	45

The amount of compounds for extraction was 100 nmol per sample. The values were the mean of duplicates compared with non-extracted standards.
Extraction methods: SPE, solid phase extraction; A, ethyl acetate; B, chloroform; C, methylene chloride; D, diethyl ether. The samples were adjusted to pH 11 with 0.3 mol l^{-1} of NaOH for methods A–D. The extracting solvent was 4 ml × 1. ND, not detectable.

As phosphate buffer (0.2 M, pH 7.4) was normally used for incubations, it was necessary to consider modifying the concentration and pH of the incubates before applying to the SPE columns. The results in Table 4 show that when the ion concentration of samples was 0.2, 0.1 or 0.025 M, respectively, the extraction recovery was not markedly changed either for BA or BANO. It was also shown (Table 4) that in the range pH 6.0–8.0, the solutions for column conditioning and washing out of interfering substances did not cause much change in the extraction recovery. It was also found that providing less than 30% MeOH was used in the solution for the washing stage it did not affect the extraction recovery significantly. However, when the ratio of MeOH in the solution was increased to 40%, part of the BANO was washed away with the interfering substances. It was also confirmed that MeOH (1 ml) was not enough to elute all analytes (Table 4). These results support the conclusion that the present procedure was appropriate.

Because of the ethical problems associated with the use of animal hepatic microsomes in blank

Table 4
The evaluation of some factors potentially affecting the extraction recovery of the SPE procedure[a]

Conditions	Value	Extraction recovery (% ± S.D.)	
		BA	BANO
Phosphate buffer concentration in sample (M)	0.025	93 ± 2.5	100 ± 4.0
	0.1	96 ± 3.9	96 ± 5.8
	0.2	94 ± 3.1	102 ± 7.8
pH value for column conditioning and interference washing	6.0	100 ± 13.7	98 ± 3.4
	7.0	96 ± 7.7	97 ± 2.2
	8.0	98 ± 4.5	100 ± 6.2
MeOH content ratio for Interference washing (%)	20	100 ± 1.5	91 ± 1.3
	30	100 ± 0.7	94 ± 2.0
	40	99 ± 1.0	53 ± 2.2
MeOH volume for analyte desorption and elution (ml)	1.0	88 ± 7.0	47 ± 4.5
	2.0	97 ± 0.4	92 ± 2.2
	3.0	100 ± 2.2	100 ± 2.3

[a] $n = 3$

experiments, a simplified procedure was adopted for establishing calibration curves without the use of animal hepatic microsomes. To evaluate the reliability of this procedure, calibration curves of 4NBA and 4NBANO were compared with or without using the inactivated microsomal mixture. Known amounts of 4NBA and 4NBANO were added to the microsomal incubation system with co-factors and incubated, extracted and analyzed according to the procedures described previously. The microsomes were inactivated by treating at 95°C for 20 min in a water bath before use. Separately, the same amounts of 4NBA and 4NBANO was added to phosphate buffer (3 ml, 0.1 M, pH 7.4) without using inactivated microsomal incubation system, extracted and analyzed with the same procedures. The results confirmed that there was no marked difference between the calibration curves obtained by these two methods. The binding between microsomal proteins and the analytes, which is one of main factors often affecting the extraction of analytes from a biological matrix, could be ignored and the simplified procedure gave reliable results (Fig. 4).

The calibration curves were linear in the range of 10–600 nmol per 1.5 ml sample for substrates and 1.25–30 nmol per 1.5 ml sample for N^1-oxides. The detectable levels of N^1-oxides that could be accordingly estimated was equal to 0.5 nmol per incubate which represents 0.5% metabolic conversion at a substrate concentration of 100 nmol per incubate.

3.3. Metabolism

The above HPLC and SPE methods were applied to studies of the in vitro metabolism of BA and NBAs by animal hepatic microsomes. Some data obtained is shown as an example of the application of the developed methods. The amount of BANO and NBANOs formed during incubation with hamster hepatic microsomes, and the amount of unmetabolized BA and NBAs present after incubation were determined. The total amount of unknown metabolites (such as dealkylation product etc. [1–3]) were obtained by subtracting the amount of formed N^1-oxides and unmetabolized substrates from the amount of added substrates (Fig. 5). The results indicated that (a) N^1-oxidation was a main metabolic pathway of BA and NBAs; (b) the order of total metabolic rate was BA > 2NBA > 4NBA and 3NBA; (c) the order of N^1-oxidation rate was 2NBA > BA > 4NBA and 3NBA; (d) the metabolic rate order for other unknown metabolites was BA > 4NBA and 3NBA > 2NBA. The detailed results will be separately reported and discussed in another publication.

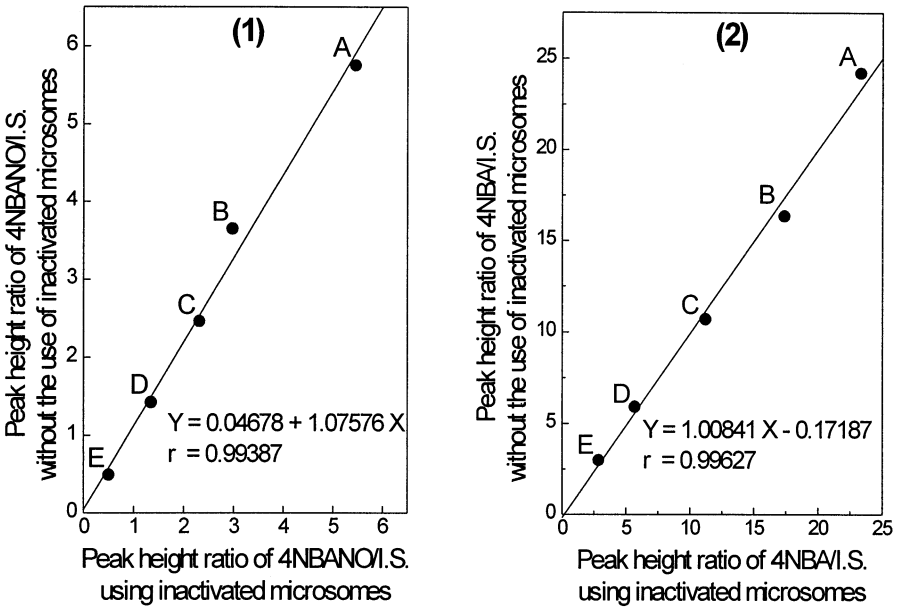

Fig. 4. Comparison of calibration curves of 4NBANO (1) and 4NBA (2) with or without inactivated hamster hepatic microsomes. The amount of 4NBANO added in samples: (A) 100 nmol; (B) 60 nmol; (C) 40 nmol; (D) 20 nmol; (E) 10 nmol. The amount of 4NBA added in samples: (A) 800 nmol; (B) 600 nmol; (C) 400 nmol; (D) 200 nmol; (E) 100 nmol.

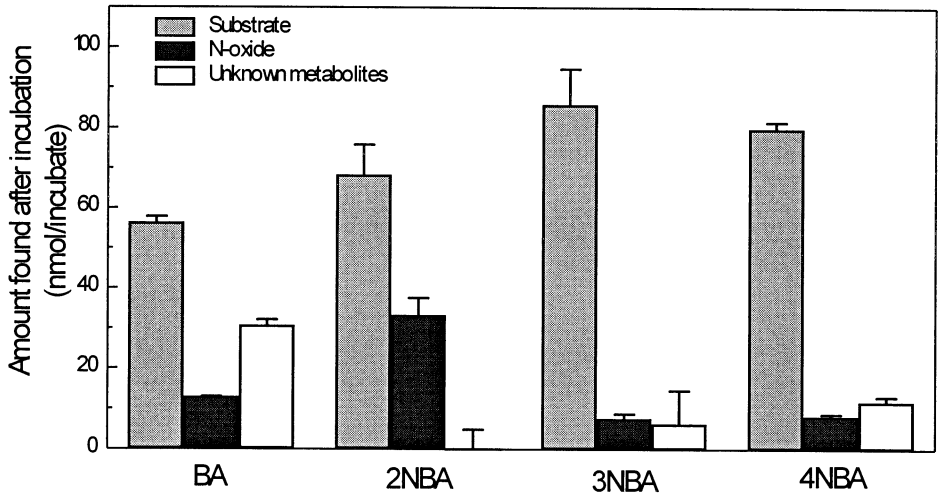

Fig. 5. The metabolism of BA and NBAs by hamster hepatic microsome ($n = 3$).

4. Conclusion

From the above, it can be concluded that the developed HPLC and SPE methods are suitable for the reliable, sensitive, rapid and convenient simultaneous determination of 9-benzyladenine or isomeric 9-(nitrobenzyl)adenines and their N^1-oxides present in hepatic incubation samples, and possibly in other biological samples. It is suggested that the methods may be valuable

in determining polar adenine derivatives and related compounds occurring in pharmaceutical products or derived from cellular macromolecules. The methods represent a successful attempt to apply a SPE method for the extraction of drugs and their metabolites from complex biological matrixes.

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