Journal of Chromatography, 619 (1993) 71–77
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7011

Liquid chromatographic determination of *para*-substituted N,N-dialkylaniline N-oxides

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(First received April 20th, 1993; revised manuscript received May 25th, 1993)

ABSTRACT

A high-performance liquid chromatographic method for the determination of *p*-substituted N,N-dialkylaniline N-oxides [N,N-dimethylaniline N-oxide (DMANO), N,N-dimethyl-*p*-toluidine N-oxide, N,N-diethylaniline N-oxide, N-ethyl-N-methylaniline N-oxide (EMANO), *p*-cyano-N,N-dimethylaniline N-oxide (pCNDMANO), and N-phenylpyrrolidine N-oxide (PPNO)] has been developed. It uses an octadecylsilica column, a mobile phase of methanol–phosphate buffer (pH 7.0, adjusted by triethylamine) and ultraviolet detection. N-Oxides were eluted in the order of hydrophilicity except for PPNO, which eluted between DMANO and EMANO. The number of theoretical plates and the detection limit under optimized conditions were between 2400 (DMANO) and 5400 (pCNDMANO) and between 2.3 μM (pCNDMANO) and 30 μM (PPNO), respectively. The concentration of N-oxides recovered from enzyme reaction mixtures by solid-phase extraction using Sep-Pak C_{18} (prior to chromatography) was also optimized with regard to the sensitivity and interference.

INTRODUCTION

Amine oxidations are important in the metabolism of both endogenous and xenobiotic chemicals [1] and are mediated in the endoplasmic reticulum by two oxygenase systems: cytochrome P450 (P450) [2] and flavin-containing monoamine oxygenase [3]. N-Oxides are recognized as one of the main products of tertiary amines [4]. N,N-Dimethylaniline (DMA) has been used as a diagnostic substrate for evaluating the metabolism of amines. Its N-oxygenation product, N,N-dimethylaniline N-oxide (DMANO), has often been quantitated using the colorimetric method developed by Ziegler and Pettit [5]. Sensitive methods were developed using gas chromatography combined with solvent extraction and $TiCl_3$ reduction by Gorrod *et al.* [6], and thin-layer chromatography (TLC) using ^{3}H-labelled DMA by Cashman [7].

We have investigated the effects of substitution of the *para*-position and the N-alkyl chain on both N-dealkylation and N-oxygenation activities of purified enzyme systems toward DMA substrates [8]. The colorimetric method [5] proved to be neither sensitive nor applicable to the *para*-substituted derivatives, and the substituted materials were not available with radio-labels. In a previous paper [8], we briefly reported analytical methods for the quantitation of *para*-substituted N,N-dialkylaniline N-oxides. This paper describes the high-performance liquid chromatographic (HPLC) methods and solid-phase extraction in detail.

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0378-4347/93/$06.00

EXPERIMENTAL

Chemicals

DMANO, N,N-dimethyl-*p*-toluidine N-oxide (DMpTNO), N,N-diethylaniline N-oxide (DEANO), N-ethyl-N-methylaniline N-oxide (EMANO), *p*-cyano-N,N-dimethylaniline N-oxide (pCNDMANO) and N-phenylpyrrolidine N-oxide (PPNO) were prepared as previously described [8]. Sep-Pak C_{18} cartridges were purchased from Waters Assoc. (Milford, MA, USA). All other chemicals were analytical-reagent grade.

Colorimetric method for N-oxides

N-Oxides were quantitated by the colorimetric method of Ziegler and Pettit [5]. A 1-ml volume of acidified supernatant of enzyme reaction mixture [P450 reconstituted system or rat liver microsomes fortified with reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) generating system (0.5 m*M* $NADP^+$, 10 m*M* glucose-6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase, 5 m*M* $MgCl_2$, 50 m*M* potassium phosphate, pH 7.7)] was mixed with 0.16 ml of 5 *M* sodium hydroxide solution and extracted three times with 3 ml of methylene chloride. An aliquot of the resulting aqueous phase was mixed with 3 *M* aqueous trichloroacetic acid (final pH 2.5), mixed with sodium nitrite (final concentration 9 m*M*) and heated for 5 min at 60°C. The absorbance was measured at 420 nm.

Solid phase extraction of N-oxides

A 2-ml volume of acidified supernatant of enzyme reaction mixture was mixed with 0.32 ml of 5 *M* aqueous NaOH and centrifugated at 3000 *g* for 5 min. The resulting supernatant was applied to a Sep-Pak C_{18} cartridge equilibrated with water. The cartridge was washed with 5 ml of water, and N-oxides were eluted with 5 ml of an aqueous methanol solution (see composition below). N-Oxides could not be quantitatively recovered after solvent evaporation without the addition of acid. Therefore, the solid-phase extract was mixed with 100 ml of 1 *M* aqueous phosphoric acid and evaporated to dryness under a nitrogen stream at 40°C to prevent any loss of N-oxides. The resulting dried sample was dissolved in 100 μl of 1 *M* aqueous NaOH, and a part of this solution was injected into the HPLC column.

Chromatography

HPLC was carried out with either a Spectra-Physics 8700 pumping system (Spectra-Physics, Piscataway, NJ, USA) equipped with an Altex 210A injector (Beckman Instruments, Berkeley, CA, USA) and a variable-wavelength detector (Spectromonitor 3100, Milton Roy), or a JASCO 800 series instrument (Japan Spectroscopic, Tokyo, Japan) equipped with a Rheodyne sample injector (Cotati, CA, USA), a 860-CO column oven and a 870 variable-wavelength detector. Quantitation was done by peak-height analysis. Various ODS columns were used, including: Inertsil ODS-2 (5 μm, 250 or 150 mm × 4.6 mm I.D., GL Science, Tokyo, Japan), YMC-PACK ODS AM302 S-5 (120 Å, 150 mm × 4.6 mm I.D., Yamamura Chemical Labs., Kyoto, Japan), TSK-Gel ODS-80T and ODS-120T (150 mm × 4.6 mm I.D., Tosoh, Tokyo, Japan), Ultracarb 5 ODS (30) (150 mm × 4.6 mm I.D., Phenomenex, Torrance, CA, USA), Zorbax ODS (3 μm, 80 mm × 6.2 mm I.D., Mac-Modd, Chadds Ford, PA, USA) and Ultremex 5 C_{18} (5 μm, 250 mm × 10 mm I.D., Phenomenex).

The mobile phase used was a mixture of methanol (HPLC grade) and aqueous buffer [25 m*M* phosphate, adjusted to pH 7.0 with triethylamine (TEA)]. TEA was distilled before use in HPLC solvents. The ratio of methanol to buffer was adjusted so that the capacity factor of the N-oxide was *ca.* 3. The column temperature was ambient (Spectra-Physics 8700 pumping system) or maintained at 40°C (JASCO 800 series instrument). The flow-rate was adjusted in the range 0.8–4.0 ml/min, depending on the separation conditions. The UV wavelength used for detection was 276 nm for pCNDMANO or 254 nm for the other N-oxides (UV maxima described previously [8]). For N-oxide analysis in enzyme reaction samples, crude components were washed from ODS columns by increasing methanol concentrations to 90% of the mobile phase after elution of N-oxides.

RESULTS AND DISCUSSION

Colorimetric assays

The colorimetric N-oxide assay [5] is based on the conversion of DMANO into *p*-nitroso-DMA, which shows strong absorbance in the visible region (λ_{max} = 420 nm, $\varepsilon \approx 10^4\ M^{-1}\ cm^{-1}$). Nitric acid reduces the N-oxide to the corresponding parent amine, and nitrosation then occurs at the *para*-position. Standard curves were linear over the DMANO concentration range 5–100 μM. Under the same conditions, DEANO and EMANO gave similar colour reactions, with a λ_{max} of 425 nm for EMANO and 430 nm for DEANO ($\varepsilon \approx 10^4\ M^{-1}\ cm^{-1}$ in both cases). However, conversion of DEANO into *p*-nitroso-N,N-diethylaniline was not complete, even with further nitric acid addition or prolonged incubation at 60°C. DMpTNO showed a weak colour reaction ($\varepsilon < 10^3\ M^{-1}\ cm^{-1}$, λ_{max} = 442 nm). pCNDMANO did not show any colour reaction.

In this method, it is also necessary first to perform organic solvent extraction to remove parent amines, which obviously show severe positive interference. As shown in Table I, part of the N-oxide fraction was extracted in the organic phase. Hydrophilic pCNDMANO, DMANO and EMANO showed more than 90% retention in the aqueous phase. In contrast, in the case of DMpTNO, PPNO and DEANO, *ca.* 20–30% of the N-oxide originally added to the aqueous phase was extracted into the organic phase. Therefore, the colorimetric method was judged not to be generally applicable to the N-oxide assay.

Solid-phase extraction

In the enzyme reaction mixture analysed for N-oxide, compounds were observed that interfered with direct HPLC. In addition, the detection sensitivity was very low (see below). Therefore, appropriate clean-up and concentration procedures are necessary prior to HPLC analysis. Solid-phase extraction has been widely used for the analysis of drugs and other chemicals, and this technique was used as a pretreatment.

The elution profiles of N-oxides from Sep-Pak C_{18} solid phase are shown in Table II. Except for pCNDMANO, N-oxides were completely retained on the Sep-Pak C_{18} cartridge when the sample solution was applied as an aqueous alkaline solution. pCNDMANO was adsorbed weakly on the solid phase, and some was eluted during sample application. The percentage recovery decreased as the sample volume increased (data not shown). By adjusting the methanol concentration for washing and elution, N-oxides could be quantitatively recovered except for pCNDMANO (Table III). At least twenty-fold concentration and clean-up of N-oxide samples could be achieved by this procedure.

TABLE I

PARTITION RATIOS OF N-OXIDES BETWEEN AQUEOUS AND ORGANIC PHASES

Known amounts of N-oxides were dissolved in 1 ml of 50 mM potassium phosphate buffer (pH 10) and extracted three times with 3 ml of methylene chloride. The organic and aqueous phases were evaporated to dryness in the presence of 100 μl of 0.1 M phosphoric acid under a nitrogen stream at 40°C and dissolved in 100 μl of 0.1 M aqueous NaOH. N-Oxides were quantitated by the HPLC method. Each value represents the percentage of N-oxide recovered in the aqueous and organic phases (mean of duplicate determinations).

	DMANO		DMpTNO		pCNDMANO		DEANO		EMANO		PPNO	
N-Oxide concentration (μM)	31	310	39	395	45	446	59	592	67	665	192	1919
Aqueous phase content (%)	94	97	73	68	91	90	78	76	92	92	89	86
Organic phase content (%)	6	3	27	32	9	10	22	24	8	8	11	14

TABLE II

DISTRIBUTION OF N-OXIDES IN ELUATES FROM SEP-PAK C_{18} CARTRIDGES

Known amounts of N-oxides (0.79–1.33 m*M*) were dissolved in 1 ml of 50 m*M* potassium phosphate buffer (pH 10) and applied to Sep-Pak C_{18} cartridges previously equilibrated with water. Elution was sequentially conducted with 2 ml of aqueous methanol. Each fraction was evaporated to dryness under a nitrogen stream at 40°C in the presence of 0.1 ml of 0.1 *M* phosphoric acid, dissolved in 100 μl of 0.1 *M* aqueous NaOH. N-Oxides were quantitated by HPLC. Each value represents the percentage of N-oxide recovered in each fraction.

Methanol (%)	N-Oxide (%)					
	DMANO	DMpTNO	pCNDMANO	DEANO	EMANO	PPNO
0	0.2	N.D.	7	2	0.7	0.3
5	14	N.D.	68	0.1	0.5	4.9
10	59	N.D.	23	0.5	30	32
20	15	56	2	29	47	52
30	9	40	N.D.	47	20	11
50	0.4	4	N.D.	19	1	0.7
100	N.D.	N.D.	N.D.	3	1	N.D.

[a] Not detected (< 0.1).

Elution profile of N-oxides from ODS columns

Chromatographic separation of *p*-substituted N,N-dialkylaniline N-oxides has been reported by Damani *et al.* [9], who described the TLC separation and detection of N,N-dialkylaniline N-oxides, and by Murray and Sligar [10], who described the HPLC separation of *p*-cyano-N,N-dimethylaniline products (substrate, *p*-cyano-N-methylaniline and pCNDMANO). However, extensive HPLC study of N-oxides has not previously been reported.

TABLE III

RECOVERY OF N-OXIDES IN SOLID-PHASE EXTRACTION

Known amounts of N-oxide (*ca.* 100 μ*M*) were added to the final reaction mixtures of rat liver microsomes (0.67 mg protein per ml) or cytochrome P450 (0.3 μ*M*) reconstituted systems fortified with an NADPH-generating system, and the mixtures were made alkaline with NaOH and applied to Sep-Pak C_{18} cartridges. After washing the cartridges with 5 ml of washing solution, N-oxides were eluted with 5 ml of eluting solution. Eluted N-oxide fractions were evaporated to dryness in the presence of 100 μl of 0.1 *M* phosphoric acid under a nitrogen stream at 40°C and dissolved in 100 μl of 0.1 *M* aqueous NaOH. N-Oxides were quantitated by HPLC. Each value represents the percentage of originally applied N-oxide recovered [mean of triplicate (indicated with ± S.D.) or duplicate determinations].

Compound	Methanol (%)		Recovery (%)	
	Washing	Elution	Microsomes	P450 system
DMANO	0	30	95.4[b]	96.7 ± 1.2
DMpTNO	0	60	96.2[b]	98.0 ± 5.2
pCNDMANO	0 (2 ml[a])	20	69.0 ± 6.4	70.9 ± 16.6
DEANO	5	60	99.6 ± 3.7	100.7 ± 6.9
EMANO	0	50	92.6 ± 9.0	105.7 ± 9.8
PPNO	0	50	94.0[b]	97.8 ± 9.5

[a] Washing with 2 ml of water.

[b] Duplicate determination.

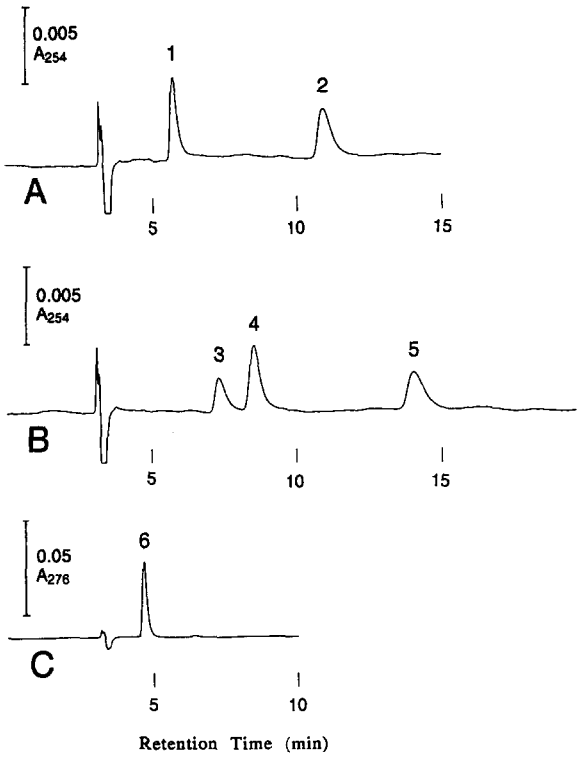

Fig. 1. Typical chromatograms of *p*-substituted N,N-dialkylaniline N-oxides. (A) Mixture of DMANO (1, 124 μM) and DMpTNO (2, 148 μM), (B) mixture of PPNO (3, 818 μM), EMANO (4, 308 μM) and DEANO (5, 181 μM) and (C) pCNDMANO (6, 221 μM) were analysed by HPLC. Elution conditions: column, Inertsil ODS-2 (250 mm × 4.6 mm I.D.); mobile phase and flow-rate, methanol–buffer (25:75, v/v) and 0.8 ml/min; column temperature, 40°C; wavelength for UV detection, 254 nm (A, B) and 276 nm (C); injection volume, 10 μl. The vertical bars indicate the absorbance scale.

Elution of N-oxides from ODS columns was performed with mobile phases consisting of aqueous solution containing low methanol concentrations. In the case of mixtures of methanol and water, N-oxide peaks were abnormally broad and showed strong tailing patterns, which may be the result of binding of N-oxides to the remaining silanol functions on silica stationary phase. In order to overcome the poor peak resolution, neutral buffer solutions containing TEA (25 mM phosphate adjusted to pH 7.0 by TEA) were examined. Because N-oxides have acidic pK_a values [11], all N-oxides are deprotonated at pH 7 and adsorb well on ODS, so TEA should suppress the non-specific binding of N-oxide. Therefore, a mobile phase containing the buffer was found to sharpen the peak shape. A typical chromatogram is shown in Fig. 1. All N-oxides were separated from each other. *p*-Cyano-substitution weakened and *p*-methyl or N-ethyl substitution strengthened the retention of N-oxides. In general, column retention was correlated with the hydrophobicity of N-oxides except for PPNO, which eluted between DMANO and EMANO, in contrast to the retention behaviour of the corresponding parent amine, N-phenylpyrrolidine, which showed the strongest retention among the parent amines. Some steric factor may contribute to the weakening of the interaction between PPNO and the ODS column.

The resolution of N-oxides varied considerably, mainly depending on the ODS columns used, as shown in Table IV. In contrast, that of the

TABLE IV

COMPARISON OF N-OXIDE SEPARATION EFFICIENCIES AMONG HPLC COLUMNS

Each value represents the number of theoretical plates (N) calculated as 5.55 × (retention time/$t_{1/2}$)^2 ($t_{1/2}$ shows peak width equivalent to half of peak height). Elution conditions were adjusted so that the capacity factor of the N-oxide was *ca.* 3.

HPLC column	DMANO	DMpTNO	pCNDMANO	DEANO	EMANO
Inertsil ODS-2	1400	1400	2700	1300	1200
TSK ODS-80T	390	720	550	1200	750
TSK ODS-120T	180	290	710	400	230
Ultracarb 5 ODS (30)	220	350	120	510	360
Ultremex 5 ODS	880	1500	2100	1700	1300
YMC ODS AM302	210	260	560	380	300
Zorbax ODS, 3 μm	230	100	1000	41	79

corresponding parent amines was fairly constant, irrespective of the ODS column used. Although the exact reason is not clear, a characteristic of ODS columns other than the reversed-phase separation mechanism may contribute to the separation efficiency of N-oxides. Therefore, the selection of an appropriate ODS column is very important in working with these compounds.

Analytical parameters of N-oxide quantitation in HPLC

Peak resolution is presented as the number of theoretical plates (N) and detection limit, at a signal-to-noise ratio (S/N) of 5, of N-oxides under the optimized HPLC conditions using Inertsil ODS-2 column and a temperature of 40°C (Table V). Although S/N = 2 has been generally used for indicating the detection limit, we adopted S/N = 5 because of the poor peak resolution. These two parameters were lower for N-oxides than those of the corresponding parent amines, owing to their low UV absorbance ($\varepsilon < 10^3\ M^{-1}\ cm^{-1}$) [8]. A calibration curve was linear for DMpTNO concentrations from 10 μM to 100 mM. The coefficients of variation (C.V.) of DMpTNO determination were 0.67% for 0.68 mM (n = 6) and 2.7% for 68 μM (n = 6), respectively. Similar C.V. values and linearities were obtained for other N-oxides (data not shown).

Interference

Low levels (< 100 μM) of DMANO were not detected in HPLC analysis of the final enzyme reaction mixture because of severe interfering peaks eluting close to the DMANO peak. Fortunately, radioisotope-labelled DMA is commercially available, and it is possible to quantitate sub-micromolar levels of metabolized DMANO by using labelled DMA and by solid-phase ex-

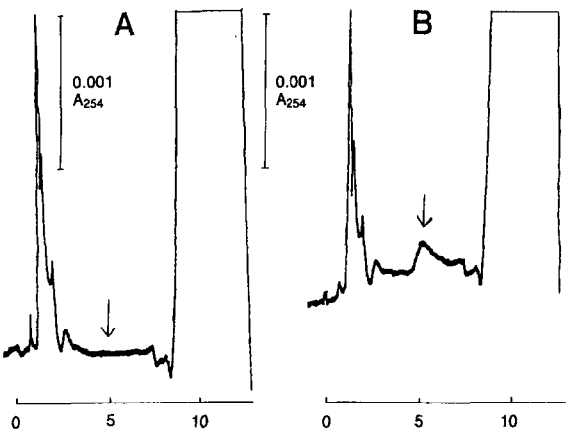

Fig. 2. Chromatograms of solid-phase extracts of the microsomal reaction mixture. A 2-ml volume of acidified enzyme reaction mixture (final 0.75 mg of rat liver microsomal protein per ml with an NADPH-generating system and 12.75% perchloric acid in the absence (A) or presence (B) of DMpTNO (14.4 μM)) was mixed with 0.32 ml of 5 M NaOH. The resulting supernatant (2 ml) was applied to a Sep-Pak C_{18} cartridge, which was washed with 5 ml of water. The DMpTNO fraction was eluted with 5 ml of 60% aqueous methanol, evaporated to dryness in the presence of 100 μl of 1 M phosphoric acid and dissolved in 100 μl of 1 M aqueous NaOH; a 10-μl aliquot was applied to the HPLC column. Elution conditions: column, Inertsil ODS-2 (150 mm × 4.6 mm I.D.); mobile phase and flow-rate: methanol–buffer (25:75, v/v) and 1.5 ml/min; column temperature, ambient; wavelength for UV detection, 254 nm. The vertical bars and arrows indicate the absorbance scale and the position at which DMpTNO eluted, respectively. The large peaks eluting near 10 min were crude components washed from the column with a mobile phase consisting of 90% methanol (v/v). The level of DMpTNO was determined to be 263 μM in the concentrated solution, and the percent recovery was 91.3%.

TABLE V

DETECTION LIMITS AND PEAK RESOLUTION OF N-OXIDE PEAKS

Elution conditions: column, Inertsil ODS-2 (250 mm × 4.6 mm I.D.); flow-rate, 0.8 ml/min; column temperature, 40°C; wavelength, 254 nm except for pCNDMANO (276 nm); injection volume, 10 ml. The ratio of methanol to buffer in the mobile phase was adjusted so that the capacity factor for each N-oxide was *ca.* 3. Detection limit indicates the N-oxide concentration needed to yield S/N = 5. Peak resolution is presented as N.

Compound	Detection limit (μM)	N
pCNDMANO	2.3	5400
DMANO	15	2400
PPNO	30	2600
EMANO	11	3200
DMpTNO	12	4200
DEANO	12	5100

traction and HPLC fractionation followed by liquid scintillation counting.

Because PPNO, EMANO, DMpTNO and DEANO are rather hydrophobic, no severe interference was observed for HPLC detection (compared with DMANO). DMpTNO was readily detected at a 10 μM level, even in the presence of a microsomal extract (Fig. 2). Therefore, low levels (> 1 μM) of these N-oxides from enzyme reaction mixtures could be detected by HPLC combined with solid-phase extraction.

Below the 10 μM level there was severe interference in the HPLC detection of the polar pCNDMANO, even with the use of high UV detection wavelength. In addition, it was not quantitatively recovered by solid-phase extraction (Table III). Therefore, it is recommended that low levels of pCNDMANO are quantitated using solvent extraction combined with $TiCl_3$ reduction, followed by HPLC analysis of the resulting pCNDMA, which shows considerably enlarged UV absorbance and is free from interference.

Application

It is possible to quantitate an appropriate level of N-oxide (> 1 μM) by HPLC analysis combined with solid-phase extraction. The overall analysis time for HPLC determination is rather short (within 20 min); elution of N-oxide (*ca.* 8 min), column washing (*ca.* 4 min) and column initialization (*ca.* 10 min), by adopting a shorter column and larger flow-rate (1.5 ml/min).

However, for more sensitive determinations, it is recommended to use this procedure (solid-phase extraction combined with HPLC) as a preparative method and treat the resulting N-oxide fraction by $TiCl_3$ reduction to form the corresponding parent amine to allow more sensitive HPLC analysis. A representative example was reported in the previous paper, describing the N-oxygenation of *p*-substituted N,N-dialkylaniline by purified rat liver cytochrome P450 2B1 [8].

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