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A new sensitive HPLC assay for methoxyamine and its analogs

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Abstract

Methoxyamine (MOA) and its analogs are polymerization regulators, building blocks and intermediates for agrichemicals and pharmaceuticals. MOA induces mutagenesis of nucleic acids and has been considered for anti-cancer and anti-virus therapy. It has been studied as a DNA repair modifier in anti-cancer therapy. HPLC procedures available in the literature for MOA are all based on electrochemical detection, which is not commonly available. This paper describes the development and validation of a HPLC assay with UV detection for MOA and its analogs. The analytes are first reacted with *o*-phthalaldehyde to form an oxime derivative before chromatography with an ODS column. Detection is achieved by UV at 254 nm. The chromatography resolves MOA from its decomposition products and analogs. The assay is reproducible (R.S.D. $< 0.8\%$), linear ($r^2 = 0.9997$), and accurate (error $< 1\%$). The method is sensitive and has a lower detection limit of 5 pmol (0.4 ng of MOA.HCl), which is comparable to that of electrochemical detection.

Keywords: Methoxyamine (MOA); Analogs; HPLC assay; Development; Validation; UV detection; OPA-oxime

1. Introduction

Methoxyamine (MOA) and its analogs have many industrial uses. They are polymerization regulators [1], especially for the so-called 'living' free radical polymerization [2,3]. They are also used as building blocks and intermediates for agrichemicals and pharmaceuticals [4–8]. MOA attacks cytosine and induces mutagenesis of nucleic acids, and has been considered for anti-cancer and anti-virus therapy [9–11]. It has been studied as a DNA repair modifier, blocking the base excision repair pathway, and a potential anti-cancer treatment [12,13]. Recently, MOA has been shown to enhance (two to threefold) the cytotoxic effect of the therapeutic methylating agent temozolomide (TMZ) and was selected by NCI's Rapid Access to Intervention Development (RAID) Program for further development [14]. Because of its chemotherapeutic use, a sim-

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ple and sensitive reversed-phase high performance liquid chromatography (HPLC) method for the assay of MOA is warranted.

MOA has been quantified with spectrophotometry [15,16], titration [17], or polarography [18,19]. Though sensitive, unlike chromatographic methods, these assays lack specificity. Sensitive and specific gas chromatographic analysis of hydroxyamine has been reported [20,21], as an oxime derivative, and can be applied to MOA. For pharmaceutical analysis in which the assay will ultimately be applied to biological fluids, aqueous based HPLC or capillary electrophoresis (CE) is more desirable. A search of the literature reveals a few HPLC [22–24] and CE [25,26] assay procedures for hydroxyamine, which may be adaptable to MOA. The CE and all except one of the HPLC methods depend on electrochemical detection. They are sensitive with a reported lower detection limit for MOA of 2 pmol [22]. Because electrochemical detectors are not commonly available, we strive for a method using the more commonly available UV detector. The only HPLC assay with UV detection reported in the literature [24] gave no detection limit. Since the detection was based on the native UV absorption of MOA at 214 nm, good sensitivity is not expected.

o-Phthalaldehyde (OPA) in the presence of excess thiol reacts with primary amines to form isoindoles which have a strong absorption maximum at 330–340 nm [27,28]. This derivatization has been widely used as a means to assay the non-chromophoric amines by HPLC using UV or fluorescent detection. Its use for hydroxyamine or alkoxyamines has not been reported, however. Since MOA also has a primary amine group, we decided to explore the feasibility of isoindole formation with OPA as a means of enhancing its UV detection for HPLC analysis. This paper describes the successful development and validation of a highly sensitive HPLC assay for MOA based on pre-column derivatization with OPA. The derivative is not the expected isoindole and its structure has been identified. The applicability of the assay to other analogs of MOA has also been demonstrated.

2. Experimental

2.1. Reagents and materials

Reagent grade potassium phosphate (KH_2PO_4) and HPLC grade MeOH were purchased from Mallinckrodt (Paris, KY). Reagent grade sodium borate (decahydrate) and *o*-phthalaldehyde (OPA) were purchased from Sigma (St. Louis, MO). 2-Mercaptoethanol (RSH) was received from Matheson Coleman and Bell (Los Angeles, CA). Solutions of 0.1 N HCl and NaOH were prepared from Dilut-it Analytical Concentrate (J.T. Baker, Phillipsburg, NJ). Acid, base and buffer solutions were prepared with H_2O purified through a Millipore Super-Q Pure Water System (Waltham, MA). Samples of MOA, as the hydrochloride salt, were purchased from Aldrich Chemicals (Milwaukee, WI), TCI Chemicals (Portland, OR), or Sigma Chemicals (St. Louis, MO). *m*-Dinitrobenzene, the internal standard, was obtained from Eastman Organic Chemicals (Rochester, NY). The internal standard solution (ISS) was prepared by dissolving 30 mg of *m*-dinitrobenzene per 100 ml methanol.

2.2. OPA derivatization

2.2.1. Procedure A (conventional)

Aliquots of 0.5 ml of analyte solutions (3–4 mM MOA in the MeOH-borate buffer mixture) were individually mixed with equal aliquots of the conventional OPA reagent (15 mM OPA in the MeOH-borate buffer which contained 1% by volume or 130 mM of RSH). The MeOH-borate buffer was a 9:1 mixture of MeOH and pH 10, 50 mM borate buffer. The combined analyte and OPA reagent solutions were homogenized and let stand at room temperature for 10 min to form the test solutions.

2.2.2. Procedure B

Aliquots of 0.5 ml of analyte solutions (7 mM MOA in the MeOH-borate buffer mixture) were individually mixed with equal aliquots of an OPA reagent (15 mM OPA in the 9:1 MeOH-borate buffer without RSH). The combined ana-

lyte and OPA reagent solutions were homogenized and heated at 80 °C for 30 min, cooled to room temperature to form the test solutions. For assay, each test solution was added with 0.5-ml aliquots of the ISS (0.30 mg ml^{-1} *m*-dinitrobenzene in MeOH) before HPLC analysis.

2.3. HPLC

The HPLC system consisted of an integrated HP 1050 system (Wilmington, DE) with an additional HP 1040 photodiode array (PDA) connected in series to the variable wavelength detector. For assay, detection wavelength was set at 254 nm, unless otherwise noted. For monitoring the UV profiles of forced decomposition products, and collection and processing of data, a HP 3D Chemstation was used. Test solutions (10 μl) were loaded onto a Phenomenex (Torrance, CA) Spherisorb ODS (2), 5 μ, 250 × 4.6 mm i.d. stainless steel column. Chromatographic analysis was carried out by isocratic elution at 1 ml min^{-1} with a 60:40 mixture of MeOH and KH_2PO_4 buffer (pH 7.0, 50 mM).

3. Results and discussion

3.1. Derivatization of MOA

When a methanolic solution of MOA was mixed with the conventional OPA–RSH reagent according to procedure A (Section 2.2.1), two tiny products **A** and **C** were formed within 10 min (Fig. 1a). When the reaction time was prolonged to 16 h, formation of **A** was continuously increased to fivefold while **C** was 40 times enhanced after 1 h and continued to increase to 80 times as the major product after 16 h (Table 1a). At the same time, another minor product, **B**, was also formed (Fig. 1b). Only product **A** has the characteristic absorption maxima (λ_{max}) at approximately 340 nm (Fig. 2a) identifiable with the expected 1-methylthio-2-methoxy-isoindole [27]. The UV spectra (Fig. 2b,c, respectively) of the other minor (**B**) and the major product (**C**) are somewhat similar to each other and to that of OPA (Fig. 2d). Apparently, formation of the isoindole (**A**) from MOA with the OPA–RSH

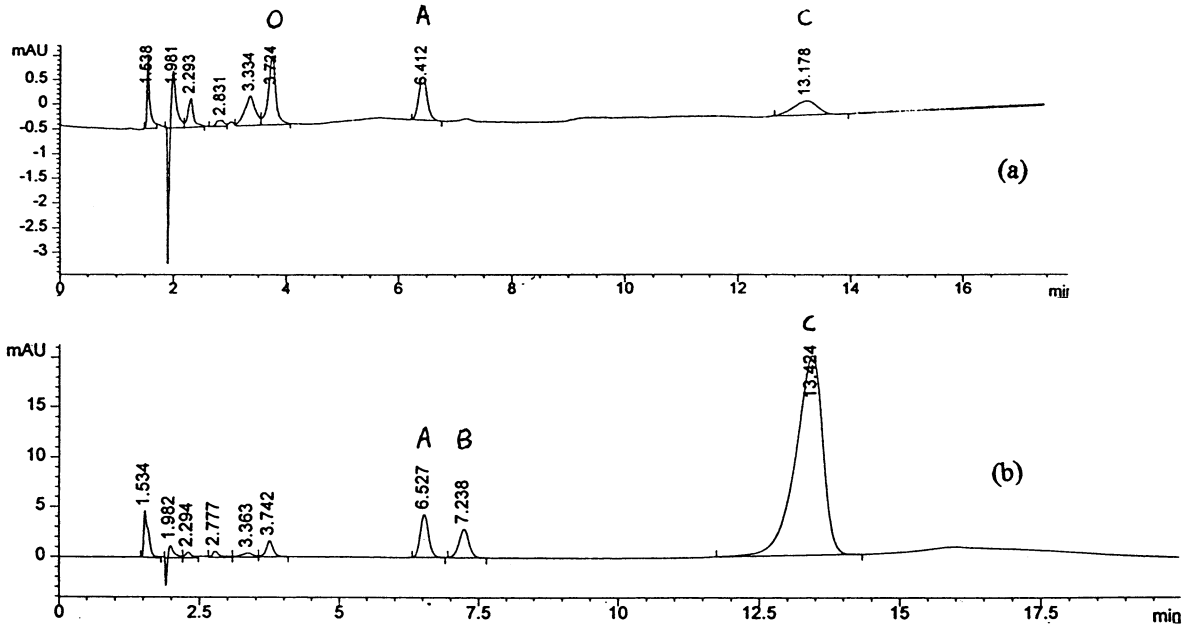


Fig. 1. HPLC profiles of a reaction mixture of MOA–OPA–RSH (molar ratio 1:4:8) at room temperature: (a) fresh solution, (b) 16 h later. HPLC conditions: Phenomenex Discovery C16 RP amide, 5 μ, 150 × 4.6 mm stainless steel column; elution with 1 ml min^{-1} of 35% MeOH in H_2O; UV detection at 330 nm.

Table 1
Formation of derivatives **A**, **B**, and **C** in constant molar ratio mixtures of MOA–OPA–RSH (1:4:8)

Reaction time (h)	Peak[a] intensity		
	A	**B**	**C**
a. Room temperature			
0	9	0	8
1	12	30	323
2	14	39	450
3	16	45	547
4	18	48	599
5.5	20	49	687
7	23	47	653
8.5	24	46	650
9	24	45	689
10.5	31	43	671
14.5	40	38	684
18	45	36	679
b. 30 °C			
0	59	17	184
0.33	62	29	310
0.67	68	42	477
1	81	49	580
c. 40 °C			
0	59	17	184
0.33	69	56	606
0.67	80	64	743
1	85	60	790

Please see Fig. 1 for HPLC conditions, Section 2.2.1 for derivatization procedure.

[a] Peak **A** = 1-methylthio-2-methoxy-isoindole; **B** = unknown; **C** = OPA–MOA oxime.

reagent is scant and slow. Increasing the reaction temperature did not improve significantly the formation of **A** (Table 1b, c). However, increasing the molar ratio of OPA–RSH reagent to MOA yielded a significant amount of **A**, apparently at the expense of **C** (Table 2a–c). But **C** still remained as the major product. The improved formation of **A** is associated with the increased presence of RSH, as a single addition of RSH to the low OPA–RSH to MOA ratio solution similarly improved the yield (Table 2d).

Since formation of the intended isoindole **A** was slow and inefficient, we turned our attention to the formation of **C**. With strong UV absorption in the 240–270-nm region, MOA can be easily detected via its derivative **C**. Data in Table 2 indicate that formation of **A** requires an excess of RSH. Conversely, **A** would not be expected if RSH is absent from the reaction mixture. Indeed, data in Table 3 confirms that, only products **B** and **C** were formed when MOA was reacted with the OPA reagent in the absence of RSH. Formation of **C** was maximized while that for **B** was minimized when the reaction mixture was heated for 30 min at 80 °C. Further heating at 80 °C did not affect the intensities of the products. Both products formed remained stable in solution for at least 18 h. Dropping the MOA–OPA molar ratio from 4:1 to 2:1 slightly improves generation of **C** and conserves the OPA reagent. Therefore, a HPLC assay by the ubiquitous UV detection is possible for MOA, after it is first derivatized as **C** according to procedure B (Section 2.2.2).

3.2. HPLC separation and assay validation

Though initial HPLC separation of the OPA derivatives of MOA (Fig. 1) was good, the peak shapes tended to lead and varied from day-to-day. To improve on the peak shapes, day-to-day reproducibility, and to accommodate the internal standard (*m*-dinitrobenzene), the HPLC process was modified as described in Section 2.3. Fig. 3a represents the typical HPLC chromatogram of an assay solution of MOA obtained according to Section 2.2.2. The main OPA derivative, **C**, is well resolved from the minor derivative (**B**), the internal standard (**IS**), and un-reacted OPA (**O**). OPA derivatives of the acid (a) and base (b) decomposition products of MOA are also separated from **B**, **C**, **IS**, and **O** (Fig. 3b,c, respectively). **C** was later (Section 3.3) identified as the OPA–MOA oxime. The minor derivative **B** remained as an unknown product of MOA and OPA. One of the acid and base degradants (labeled **H**) co-eluted with and was identified as the OPA derivative of hydroxyamine. Hydroxyamine is an anticipated hydrolytic product of MOA. OPA derivatives of other alkoxyamines were also separated from one another and from that of MOA or from the **IS** (Fig. 4). Hence, the HPLC assay developed for MOA can be applicable to its analogs.

Validation of the HPLC assay for MOA was based on chromatographic data derived from its

standard solutions. The reproducibility (R.S.D.) of the combined derivatization and chromatography process, obtained from six different standard sample solutions of similar MOA concentration (4–10 mM), was 0.4–0.8% within day and 2.2% between days ($n = 4$). Based on data from five standard solutions of 0.4–0.8 mg ml^{-1} or 5–10 mM of MOA.HCl (Table 4), the assay was linear ($r^2 = 0.9997$, slope = 4.1267 with standard error of 0.0379, and intercept = 0.0172 with standard error of 0.0126) and accurate (error = 0.4%). Based on a 3:1 signal-to-noise ratio, the lower detection limit for MOA.HCl was 0.4 ng or 5 pmol which is comparable (2.5 times) to that (2 pmol) of electrochemical detection [22]. Accuracy of the assay was further demonstrated by the analysis of three lots of MOA.HCl. The purity were established as 98.5 ± 1.0% for lot A (Sigma),

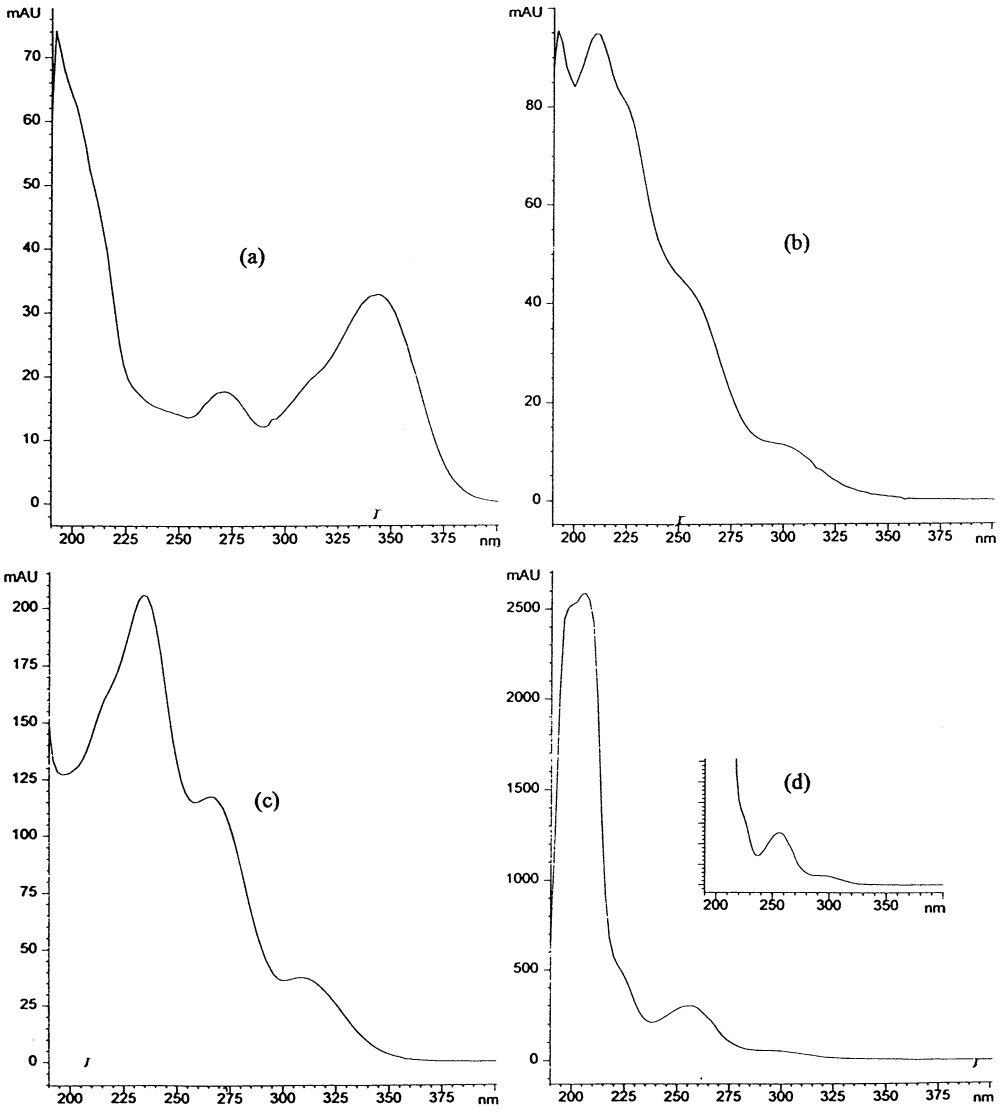


Fig. 2. UV spectra of MOA–OPA–RSH derivatives (a) **A**, (b) **B**, (c) **C**, and (d) unreacted OPA, obtained by HPLC–PDA. HPLC condition is described in Fig. 1.

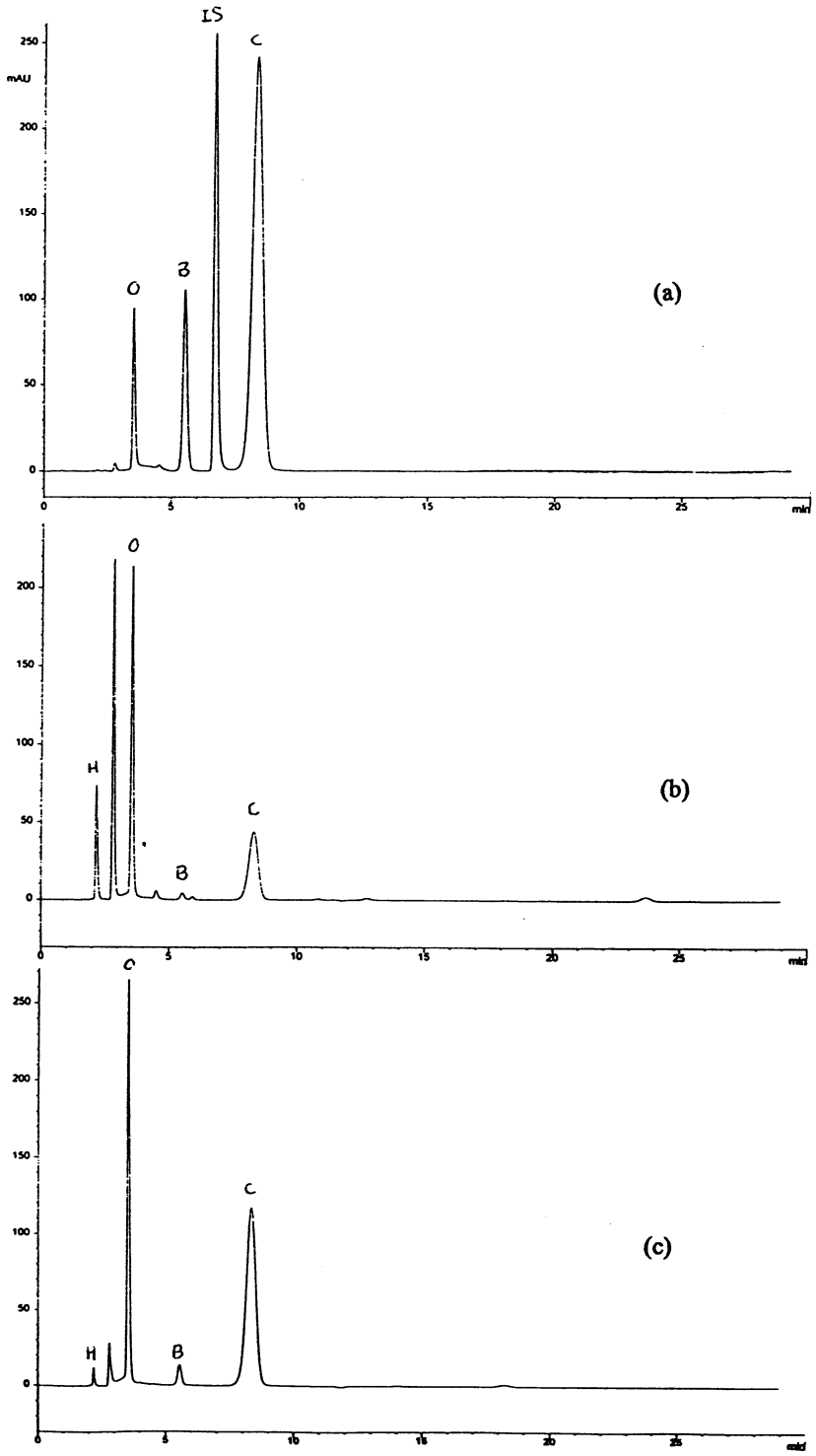


Fig. 3. HPLC profiles of (a) an assay solution of MOA prepared according to Section 2.2.2; (b) a test solution of MOA after the MOA was first heated for 30 min at 80 °C in MeOH–0.1 N HCl, followed by neutralization with 0.1 N NaOH, before OPA derivatization, and (c) a test solution of MOA after the MOA was first heated for 30 min at 80 °C in MeOH–0.1 N NaOH, 1:1 before derivatization. See Section 2.3 for HPLC conditions.

Table 2
Formation of **A**, **B**, and **C** from different molar ratio mixtures of MOA–OPA–RSH at room temperature

Reaction time (h)	Peak[a] intensity		
	A	**B**	**C**
a. Molar ratio of 1:4:8			
0	9	0	8
1	12	30	323
2	14	39	450
3	16	45	547
4	18	48	599
5.5	20	49	687
7	23	47	653
8.5	24	46	650
9	24	45	689
18	45	36	679
b. Molar ratio of 1:10:21			
0	21	0	0
1	23	16	165
2	26	28	300
3	29	36	400
4	36	43	500
5.5	37	44	526
7	44	46	580
8.5	51	46	608
9	58	44	628
18	105	31	649
c. Molar ratio of 1:21:42			
0	82	0	0
1	89	12	132
2	99	21	235
3	108	28	320
4	114	33	390
5.5	130	37	456
7	143	39	501
8.5	159	40	530
9	180	39	547
18	337	28	570
d. Molar ratio of 1:4:42			
0	42	0	0
1.5	149	18	194
3	242	27	308
4	331	33	387
5.5	416	35	432
7	499	36	460
8.5	582	35	475
10	668	33	490

Please see Fig. 1 for HPLC conditions, Section 2.2.1 for derivatization procedure.

[a] See footnote in Table 1 for peak identity.

$98.4 \pm 1.0\%$ for lot B (TCI), and $98.4 \pm 1.0\%$ for lot C (Aldrich) by a consideration of their NMR, TGA, HPLC data, and NMR assay. The HPLC assay for lots B and C, using the 98.5% pure lot A as the working standard, are 97.9% ($s = 0.7\%$, $n = 5$) and 98.3% ($s = 0.7\%$, $n = 5$), respectively. The assay results are within experimental error of those derived from NMR assays.

The HPLC assay was also partially validated for analogs of MOA. Table 5 presents the repeatability, linearity, and accuracy data of HPLC assays of *t*-butoxyamine (BuOA), benzyloxyamine (BzOA), 4-nitrobenzyloxyamine (NBOA), and tetrahydro-2H-pyran-2-yloxyamine (TPOA). Hydroxyamine, whose OPA derivative is eluted near the solvent front (3 min), was not validated for this HPLC assay.

3.3. Identification of the OPA-MOA derivative

Effort to identify the OPA–MOA derivatives was focussed on **C**, since it is the major derivative (often $> 90\%$). The UV profile of **C** (Fig. 2c) bears resemblance to that of OPA (Fig. 2d). The λ_{max} of **C** at 265 and 310 nm have a red shift of approximately 10 nm from those of OPA. This suggests that **C** is likely an OPA oxime of MOA, since λ_{max} values of oximes are known to have a red shift of approximately 5 nm from the parent aldehydes [29]. LC-MS of **C** (m/e: 164, 132, 119,

Table 3
Formation of **A**, **B**, and **C** in mixtures of MOA–OPA (molar ratio of 1:4; no RSH)

Temperature (°C)	Reaction time	Peak[a] intensity		
	(h)	**A**	**B**	**C**
23	0	0	0	18
	1	0	33	350
	4	0	56	812
	7	0	54	827
	9.5	0	52	847
40	0.5	0	61	740
60	0.5	0	49	837
80	0.5	0	18	871
	0.75	0	20	878
	1.0	0	20	847

Please see Fig. 1 for HPLC conditions.

[a] See footnote in Table 1 for peak identity.

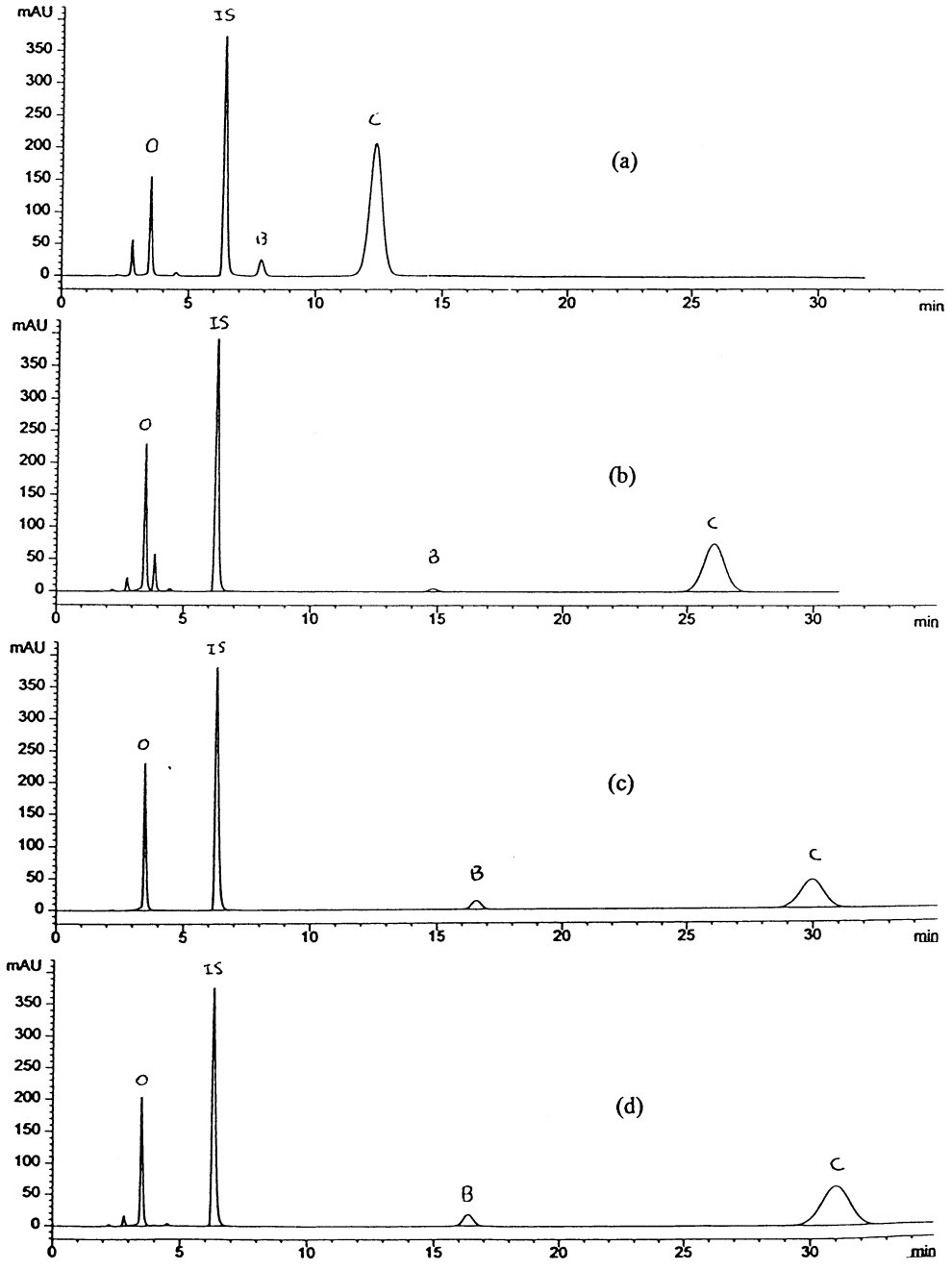


Fig. 4. HPLC profiles of assay solutions of (a) tetrahydro-2H-pyran-2-yloxyamine, TPOA; (b) 4-nitrobenzyloxyamine, NBOA; (c) butoxyamine, BuOA; and (d) benzyloxyamine, TPOA. See Section 2.2.2 for sample preparation and Section 2.3 for HPLC details.

Table 4
Linearity and accuracy data for HPLC validation of MOA assay

Standard	Peak intensity			mg ml^{-1} of MOA.HCl		Error[b]
	MOA	IS	F = MOA/IS	Actual	Found[a]	%
1	3803	2282	1.6661	0.4051	0.4079	+0.69
2	4757	2265	2.1003	0.5144	0.5131	−0.25
3	5583	2271	2.4588	0.6020	0.6000	−0.33
4	6394	2234	2.8619	0.7002	0.6977	−0.36
5	7692	2231	3.4482	0.8368	0.8398	+0.36
						Avg[c] 0.40

See Section 2.2.2 for sample preparation and Section 2.3 for HPLC conditions. Linear regression analysis of $F(x)$ vs. actual MOA concentration (y) gave $y = 4.1267x - 0.0172$, $r^2 = 0.9997$. Standard error of slope and intercept were 0.0379 and 0.0126, respectively.

[a] Found MOA concentration = $(F + 0.0172)/4.1267$.

[b] Error = (found MOA − actual MOA)/actual MOA concentration × 100.

[c] Avg = $\Sigma|\text{error}|/5$.

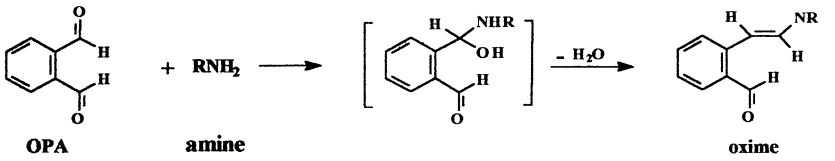
101) indicates that it has a molecular weight of 163, which is consistent with the structure of the OPA–MOA oxime (Fig. 5). Oxime is formed by dehydration of the carbinolamine intermediate resulted from the addition of a primary amine to the aldehyde as depicted in Fig. 5, Scheme 1. NMR study of the OPA–MOA derivatization process suggests a more complicated process. Fig. 6a is the ^{1}H-NMR spectrum of OPA in $CDCl_3$. The two aldehyde protons, as expected, are observed as a singlet at 10.5 ppm. The aryl proton signals appear as two groups, each for two protons, at 7.8 and 8.0 ppm. When the spectrum was obtained from a CD_3OD or CD_3OD-D_2O (1:1) solution, they are identical to each other (Fig. 6b) but markedly different from the $CDCl_3$ spectrum. The aryl signals are slightly unshielded and collapsed to a single group at 7.4 ppm. More interestingly, the aldehyde singlet is nearly gone, in its place are four unequal singlets at 6.0–6.5 ppm. These signals are identical to those reported for 1,3-dimethoxy-1,3-dihydroisobenzofuran, HBF [27]. Apparently, in protic solvents such as MeOH or HOH, OPA does not exist as the aldehyde. It exists primarily as HBF via intermediate I (Scheme 2, Fig. 5). Fig. 7a is the NMR spectrum of a test solution of OPA–MOA prepared with deuterated solvents according to Section 2.2.2 before the 80 °C heating. The accompanying HPLC profile indicates that little, if at all, derivatization has taken place. The NMR spectrum shows clearly that OPA exists as the HBF with the aryl protons at 7.4 ppm and the acetalic protons as singlets at 5.7–6.3 ppm. After the 80 °C heating, formation of primarily **C** is evident by HPLC while new NMR signals emerge at 7.7, 7.9, 8.9, and 10.3 ppm at the expense of the HBF signals (Fig. 7b). These signals suggest that the structure of derivative **C** is consistent with the OPA–MOA oxime: 7.7 (2 aryl H), 7.9 (2 aryl H), 8.9 (imino CH), and 10.3 ppm (aldehyde H).

The NMR experiments suggest, among other possibilities, that a small amount of intermediate I may exist at equilibrium with HBF and intermediate II, the mono-aldehyde (Scheme 2, Fig. 5). MOA or heating may have shifted the equilibrium to

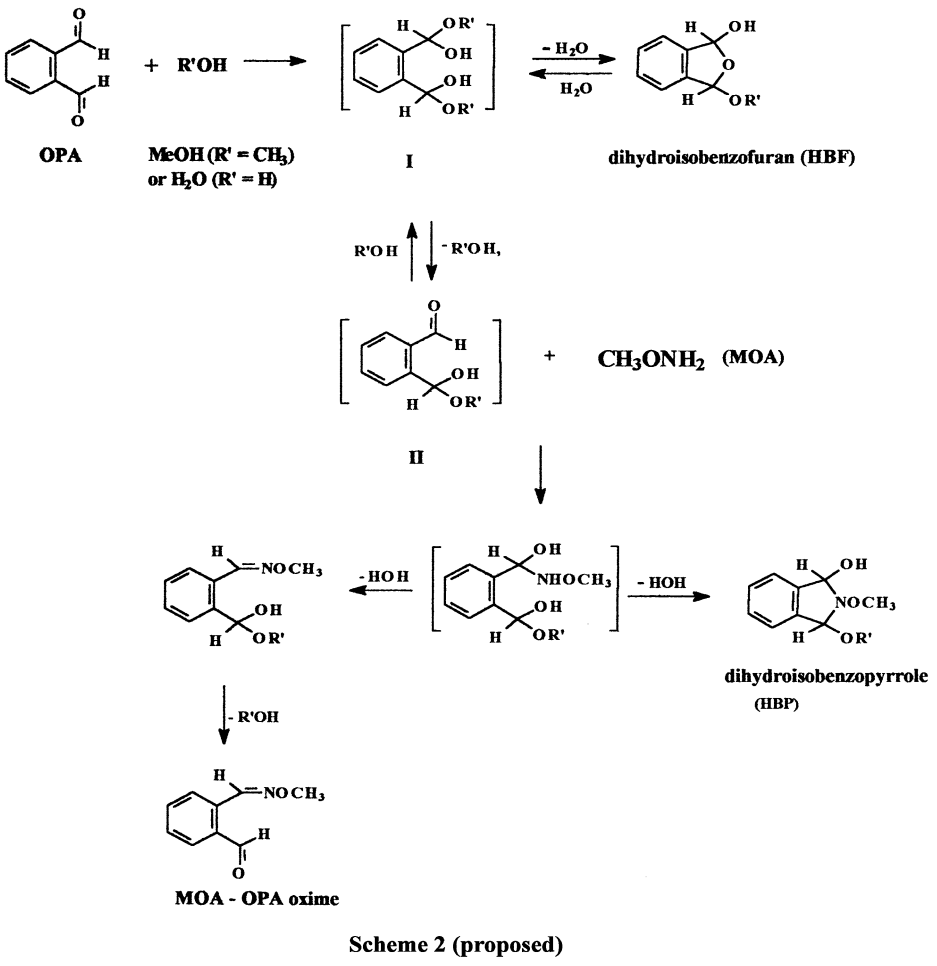
Table 5
Assay validation results for analogs of MOA ($n = 5$)

Sample[a]	r^2	Slope	Intercept	Error
BuOA	0.9996	2.8814 ± 0.0339	+0.0310 ± 0.0165	0.78%
BzOA	0.9997	2.4095 ± 0.0287	−0.0366 ± 0.0130	0.75%
NBOA	0.9998	2.7951 ± 0.0225	−0.0634 ± 0.0187	0.74%
TPOA	0.9998	2.5402 ± 0.0197	−0.0067 ± 0.0107	0.47%

[a] See Fig. 4 for abbreviations.



Scheme 1 (conventional)



Scheme 2 (proposed)

Fig. 5. Conventional and proposed mechanism of oxime formation between OPA and MOA.

intermediate II. The aldehyde in II reacts with MOA, followed by H_2O and CH_3OH elimination, and forms the OPA–MOA oxime as depicted in Scheme 2 (Fig. 5). In this scenario, a dihydroisobenzopyrole (HBP) can also be formed, though its MS and NMR data would be different from those of **C**. When the reaction was carried with solutions of OPA and MOA at higher concentrations (> 5 times), a late eluting product with MS consistent with the dioxime where both aldehyde group of OPA were derivatized with MOA (data not shown).

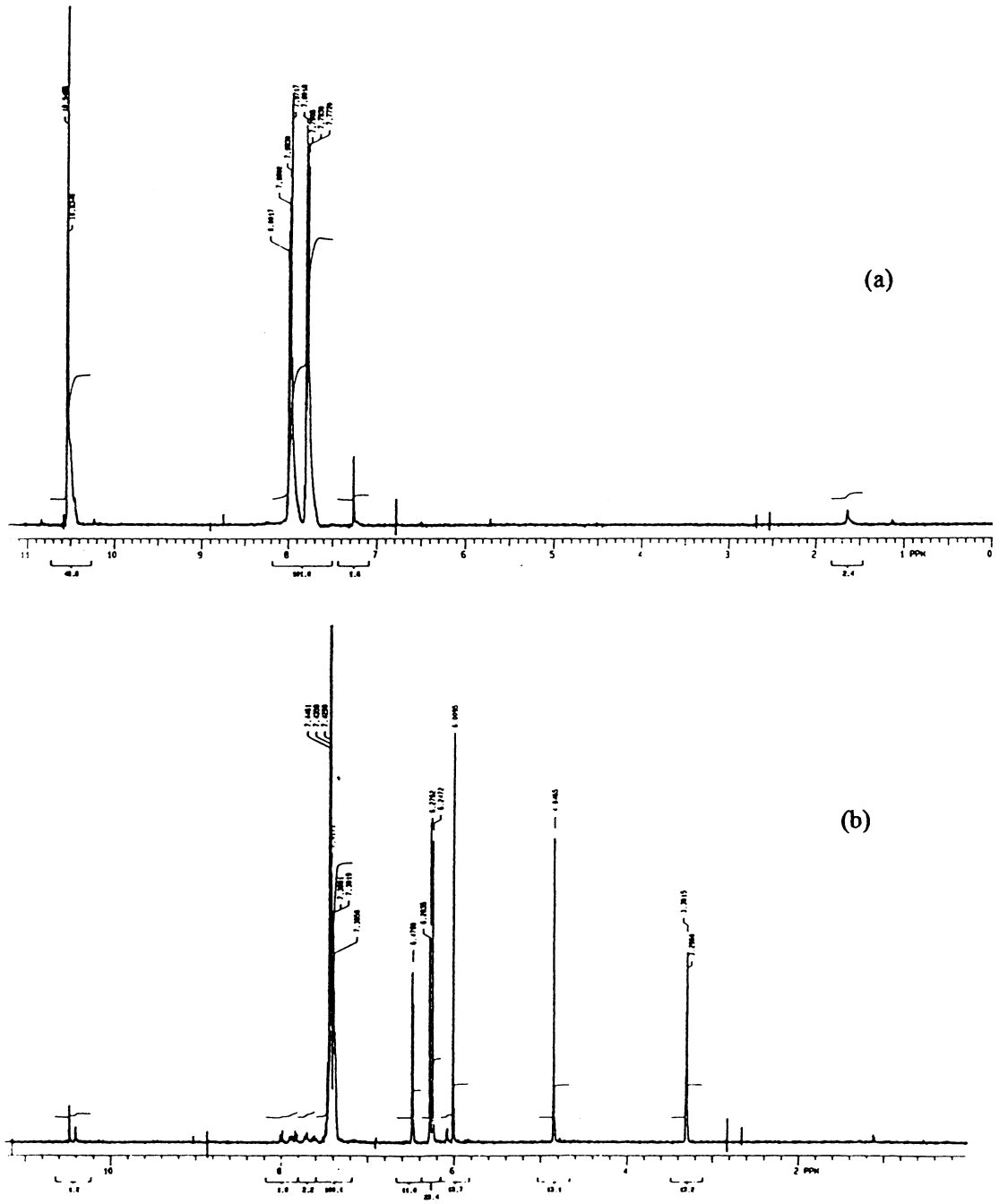


Fig. 6. ^{1}H-NMR spectra of OPA in (a) $CDCl_3$ and (b) CD_3OD.

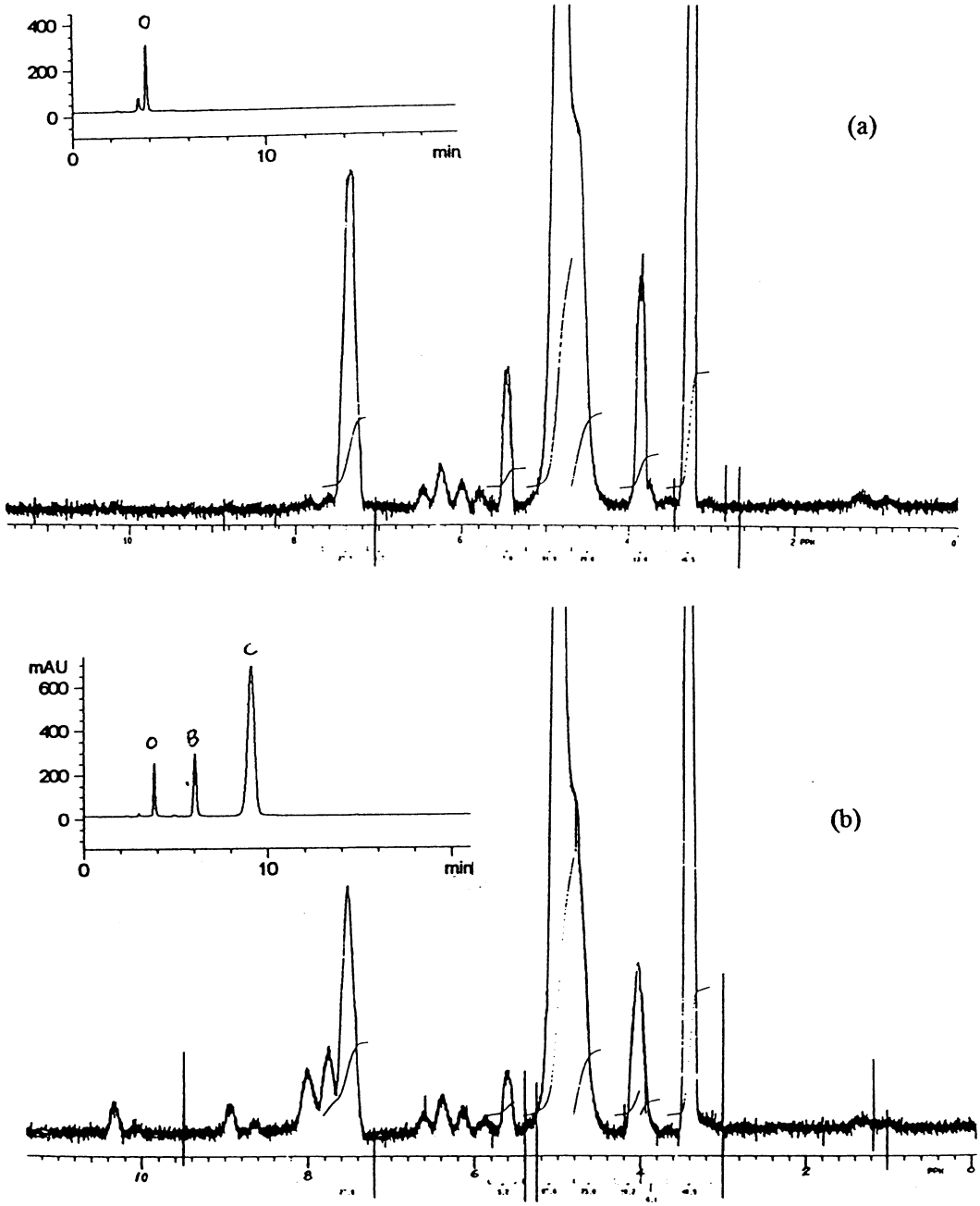


Fig. 7. [1]H-NMR spectra of a MOA–OPA solution, prepared according to Section 2.2.2 with all deuterated solvents: (a) before heating and (b) after heating at 80 °C for 30 min. Inserts are the respective HPLC profiles of the solutions, obtained according to Section 2.3.

UV profile of the minor derivative **B** (Fig. 2b) is also similar to that of OPA. But it was either too weak or not volatile enough for LC-MS; it remains an unknown derivative. Analogous minor products, with HPLC retention changes similar to those of the major product, were similarly formed from analogs of MOA (Fig. 4). Therefore, **B** must be a product of the alkoxyamine and OPA.

4. Conclusion

A HPLC assay with UV detection for MOA has been developed and validated. The assay is specific and is based on the formation of an OPA–MOA oxime before HPLC separation. It resolves MOA from analogs and potential decomposition products. The derivatization and HPLC process are precise, having a within-day R.S.D. of 0.8% and a day-to-day R.S.D. of 2.2%. The assay has a lower detection limit of 5 pmol of MOA, which is comparable to that of electrochemical detection. NMR study indicates that OPA exists primarily as 1,3-dimethoxy-1,3-dihydroisobenzofuran (HBF) in MeOH or aqueous MeOH. The HBF probably first reverts back to an aldehyde before reaction with MOA to form the OPA–MOA oxime.

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