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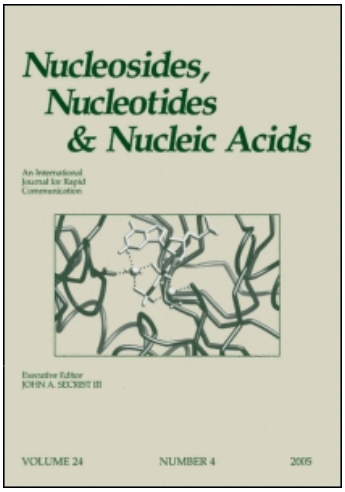

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WHAT ARE THE PRACTICAL LIMITS FOR DETECTION OF MINOR NUCLEOSIDE REACTION PRODUCTS WITH HPLC (UV DETECTION), ^{1}H NMR, AND TLC (UV DETECTION)?[1]

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ABSTRACT: Detection limits for the minor component in binary mixtures of Ado/AraA, Ado/XyloA, and Urd/dUrd depend strongly on the combined concentration of analytes. Limiting concentrations (in which ≤1% of the minor component was detected) were about two orders of magnitude lower with HPLC (UV detection) than with ^{1}H NMR and TLC (UV detection) with these nucleosides (ε_{max} 10 000–15 000). Minimum molar percentages of minor components detected in the 0.1–10 mM range were 0.25–1% with HPLC (UV), 1–2% with ^{1}H NMR, and ~2% with TLC (UV).

Introduction

During our recent studies on stereoselective reductions of 2'- and 3'-ketonucleoside derivatives,[2] uncertainties existed in the levels of semi-quantitative estimation of product ratios (especially when one diastereomer was highly predominant). Discrepancies in reported ratios of reduced nucleoside diastereomers[3-9] have resulted from the lack of control of purities of substrate ketonucleosides,[2] and also from uncertainties in the limits of semi-quantitative analytical methods used routinely in synthetic organic chemistry. Authors have claimed complete stereoselectivity (or stereospecificity) for reactions in cases in which ^{1}H NMR spectra failed to reveal visible (or integrated) levels of minor stereoisomers,[6] and difficulties in the evaluation of such ratios have been reported.[9] A recent communication noted an "estimated detection limit 1–2%" for "analyses by ^{1}H NMR and ^{19}F NMR" of fluorinated organostannanes.[10]

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We now report studies in which three accurately measured binary mixtures of nucleosides (two diastereomer pairs and one ribonucleoside/2'-deoxynucleoside pair) were evaluated by HPLC (UV detection), ^{1}H NMR, and TLC (visual UV detection) to determine semi-quantitative limits for detection of the minor component. The C2' epimers adenosine (Ado, **1**; Figure 1) and 9-(β-D-arabinofuranosyl)adenine (AraA, **2**), the C3' epimers Ado (**1**) and 9-(β-D-xylofuranosyl)adenine (XyloA, **3**), and the paired uridine (Urd, **4**) and 2'-deoxyuridine (dUrd, **5**) were selected as representative models.

Results and Discussion

HPLC with integrated UV detection at 254 nm [λ_{max} ~260 nm for all of these nucleosides (ε_{max} ~15 000 for adenine nucleosides; ε_{max} ~10 000 for uracil nucleosides)] allowed minimum levels of detection of ~0.25% (at combined analyte concentrations of 5.24×10^{-4} M; *e.g.*, **1** + **2**) for the minor component when it was eluted prior to the major component (Table 1). Minor components at 0.1% were not detected (*i.e.*, no peak integration at the control retention time). In contrast, the level of detection of minor components was raised to ~1% when the major component was eluted first, presumably resulting from "tailing" of the major component (0.5% of minor components were not detected as second elutants). Integrated UV-detection limits by relative percentages obviously depend on the combined concentrations of analytes in the mixture (Beer's law). Thus, 3% of **2** (eluted first) was not detected in a mixture (**1/2**, 97:3) with a combined concentration of 1.31×10^{-5} M (Table 2).

Minimum detection levels of the minor component with ^{1}H NMR were ~1% (500 MHz) or 1-2% (200 MHz) with the same number of scans (*e.g.*, combined concentration of **1** + **2** = 8.38×10^{-3} M) by integration of one-proton peaks for the anomeric (H1') protons of **1** [δ 5.89 (d, J = 6.1 Hz)] and **2** [δ 6.26 (d, J = 4.1 Hz)]; or **4** [δ 5.79 (d, J = 5.3 Hz)] and **5** [δ 6.17 (t, J = 6.8 Hz)]; and one-proton multiplets for the H2' protons of **1** [δ 4.62 (m)] and **3** [δ 4.33 (br s)]. ^{1}H NMR data were in agreement with those reported for 3'[^{2}H]-**1**,[2] **2**,[4] **3**,[5] **4**,[11] and **5**.[11] Signals that were visually difficult to differentiate from noise, and which were not integrated, were defined as below the limit of detection. The concentration effect resulted in lack of detection of 3% of **2** in a mixture (**1/2**, 97:3) with a combined concentration of 1.18×10^{-3} M. The ^{1}H NMR detection limits for relative percentages of components in these binary mixtures are much more sensitive to the combined concentration of analytes than to the number of scans or the field strength of the spectrometer.

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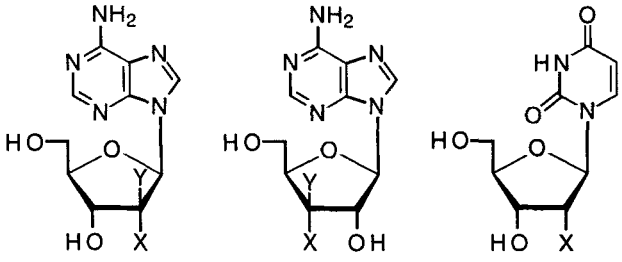

FIG. 1. Structures of nucleosides investigated.

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TABLE 1. Approximate Detection Limits by HPLC and ^{1}H NMR.

compound mixtures	HPLC (UV)	^{1}H NMR		combined conc. (M)
		200 MHz[a]	500 MHz[b]	
1 (major) **2** (minor)	0.25%[c]	1%	1%	5.24×10^{-4} [d]
1 (minor) **2** (major)	1%[e]	2%	1%	8.38×10^{-3} [f]
1 (major) **3** (minor)	0.25%[c]	1%	1%	5.24×10^{-4} [d]
1 (minor) **3** (major)	1%[e]	2%	1%	8.38×10^{-3} [f]
4 (minor) **5** (major)	0.25%[c]	2%	2%	5.73×10^{-4} [d]
4 (major) **5** (minor)	1%[e]	1%	1%	9.17×10^{-3} [f]

[a] 300 scans. [b] 64 scans (300 scans gave comparable detection).
[c] Eluted first. [d] HPLC. [e] Eluted second. [f] ^{1}H NMR.

TABLE 2. Concentration Effects on Detection Limits for **1/2** (97:3) by HPLC and ^{1}H NMR.

combined conc. (M)	HPLC (UV)	combined conc. (M)	^{1}H NMR (200 MHz)
1.31×10^{-3}	*a*	1.18×10^{-2}	*a*
1.31×10^{-4}	*a*	1.18×10^{-3}	*b*
1.31×10^{-5}	*b*	1.18×10^{-4}	*c*
1.31×10^{-6}	*c*	1.18×10^{-5}	*c*

[a] Both compounds detected. [b] The minor compound not detected. [c] Neither compound detected.

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TLC analysis [UV (254 nm), visual observation] of the Urd/dUrd pair indicated a practical detection limit of ~2% of the minor component (application of 5.0 μL of solutions with combined analyte concentrations of 2.29×10^{-3} M). Tentative shadows for the minor component "spots" were observed with mixtures of **4/5** (99:1 and 1:99) with a "dark spot" for the major components. Obviously, the volumes of solutions applied and the combined concentrations of analytes control these detection levels, but estimations of ~2% of minor components can be made by routine TLC.

In summary, semi-quantitative detection limits for the minor component in binary mixtures of Ado/AraA, Ado/XyloA, and Urd/dUrd depend strongly on the combined concentration of analytes. Limiting combined concentrations (in which ≤1% of the minor component was detected) were about two orders of magnitude lower with HPLC (UV) than with ^{1}H NMR for these nucleosides (ε_{max} 10 000–15 000). Minimum molar percentages detected for minor components at "typical" concentrations (Tables 1 and 2) were 0.25–1% with HPLC and 1–2% with ^{1}H NMR.

Experimental Section

Analytical RP-HPLC was performed with a Spectra Physics SP 8800 ternary pump system and a Dynamax C_{18} column (UV detection at 254 nm with a Hewlett Packard HP 3396 integrator). TLC was performed with Merck Kieselgel 60 F_{254} sheets (visual observation under 254 nm light). ^{1}H NMR spectra were recorded at 200 or 500 MHz (Varian Gemini 200 or VXR 500S spectrometers) with solutions in TMS/Me_2SO-d_6 (with

64 or 300 scans for each sample). Adenosine (Ado, **1**), 9-(β-D-arabinofuranosyl)adenine (AraA, **2**), uridine (Urd, **4**), and 2'-deoxyuridine (dUrd, **5**) were commercial samples. XyloA [9-(β-D-xylofuranosyl)adenine, **3**; data as reported[5]] was prepared (60% overall) by coupling adenine with 1,2,3,5-tetra-*O*-acetyl-D-xylofuranose ($SnCl_4/CH_3CN$)[12] and deprotection (NH_3/MeOH). HPLC-grade H_2O and CH_3CN were obtained from Fisher.

Sample Preparation. Stock solutions (1.31×10^{-3} M) of Ado (**1**), AraA (**2**), and XyloA (**3**) were prepared by dissolving each nucleoside (35.0 mg) in HPLC-grade H_2O (100 mL). Aliquots were combined and diluted (10.0 → 25.0 mL) to give combined concentrations of 5.24×10^{-4} M for mixtures of **1/2** and **1/3** with individual molar ratios (99.9:0.1, 99.75:0.25, 99.5:0.5, 99:1, 98:2, 97:3, 95:5, 90:10, 50:50, 10:90, 5:95, 3:97, 2:98, 1:99, 0.5:99.5, 0.25:99.75, 0.1:99.9). Stock solutions of Urd (**4**, 35.0 mg) and dUrd (**5**, 32.7 mg) were prepared similarly (concentrations of 1.43×10^{-3} M). Combined aliquots were diluted to give combined concentrations of 5.73×10^{-4} M of **4/5** with various molar ratios (99.9:0.1–97:3 and 3:97–0.1:99.9). Successive ten-fold dilutions of **1/2** (97:3; 1.31×10^{-3} M) gave combined concentrations of 1.31×10^{-4}, 10^{-5}, and 10^{-6} M.

HPLC Analyses. The column was conditioned for ~30 min with H_2O/CH_3CN (93.5:6.5, v/v, when the minor component was eluted first; or 95.5:4.5, when it was eluted second) for **1/2** and **1/3**. The different solvent ratio reduced tailing when the major component was eluted first. H_2O/CH_3CN (98:2) was used for **4/5** when **4** was the minor component (eluted first), and (99:1) when **5** was the minor component (eluted second). Solvents were degassed and then maintained under He before and during analyses. HPLC solvent systems were calibrated with individual samples of **1–5** and 1:1 mixtures of **1/2**, **1/3**, and **4/5**: **1** [t_R = 9.5 min (93.5:6.5), t_R = 16.5 min (95.5:4.5)], **2** [t_R = 7.6 min (93.5:6.5), t_R = 12.8 min (95.5:4.5)], **3** [t_R = 8.1 min (93.5:6.5), t_R = 14.0 min (95.5:4.5)], **4** [t_R = 5.2 min (98:2), t_R = 5.8 min (99:1)], **5** [t_R = 6.4 min (98:2), t_R = 7.3 min (99:1)]. Injected solution volumes (20 μL) and flow rates (1.0 mL/min) were kept constant with isocratic conditions at ambient temperature. The column was conditioned for 10 min with the new solvent system after each run. Integrated areas for separated peaks on chromatograms had ratios within ±5% of values calculated from the prepared molar ratios.

NMR Analyses. Aliquots (16.0 mL, containing 2.24 mg) of solutions of the mixtures of **1/2** and **1/3** (combined concentrations 5.24×10^{-4} M) or **4/5** (5.73×10^{-4} M) were evaporated. The dried residues were dissolved (Me_2SO-d_6, 1.0 mL) to give combined concentrations of 8.38×10^{-3} M for **1/3** and **1/4** mixtures, and 9.17×10^{-3} M for **4/5**. Analogous aliquots (9.0 mL, 3.15 mg) of **1/2** (97:3; 1.31×10^{-3} M) provided concentrations of 1.18×10^{-2} M, and ten-fold dilutions were prepared from other **1/2** mixtures.

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TLC Analyses. Various molar ratios of **4/5** (99.9:0.1–97:3 and 3:97–0.1:99.1) were analyzed by TLC [MeOH/$CHCl_3$ (1:4); **4** (R_f 0.28), **5** (R_f 0.42)]. Aliquots (6.0 mL) of **4/5** solution mixtures (combined concentrations of 5.73×10^{-4} M) were evaporated and residues were dissolved (MeOH; 0.30, 1.5, or 3.0 mL) to give combined concentrations of 1.15×10^{-2}, 2.29×10^{-3}, or 1.15×10^{-3} M, respectively. Aliquots (5.0 or 10.0 μL) of these solutions were applied to the baseline of a TLC sheet.

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