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Letters

Rapid Enantiomeric Quantification of an Antiviral Nucleoside Agent (D,L-FMAU, 2′-Fluoro-5-methyl-β,D,L-arabinofuranosyluracil) by Mass Spectrometry

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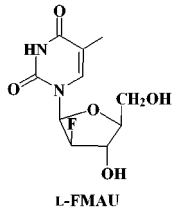
Abstract: A novel mass spectrometric method is applied to rapid, accurate (<1%) quantification of chiral Clevudine (L-FMAU, 2′-fluoro-5-methyl-β,L-arabinofuranosyluracil), a potent antiviral nucleoside agent against hepatitis B virus. Transition metal bound complex ions containing the chiral drug are generated by electrospray ionization mass spectrometry and subjected to collision-induced dissociation. The ratio of the two competitive dissociation rates is related to the enantiomeric composition of the drug mixture, allowing the determination of enantiomeric contamination in the drug.

A number of unnatural L-nucleoside analogues are being investigated as potent chemotherapeutic agents against human immunodeficiency virus (HIV),[1] hepatitis B virus (HBV),[2] and certain forms of cancer.[3] Their enantiomeric counterparts, the D-nucleoside analogues, usually show substantial toxicity likely due to their incorporation into cellular chromosomal and/or mitochondrial DNA.[4] Clevudine (L-FMAU, 2′-fluoro-5-methyl-β,L-arabinofuranosyluracil, **1**), for instance, is currently in clinical trials as a potent anti-HBV agent,[5] with EC_{50} as low as 0.13 μM and no indication of any apparent clinical toxicity, including interference with mitochondrial function. Its enantiomer, D-FMAU, exhibits unacceptable toxicity in preclinical trials.[4]



L-FMAU

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The case of D,L-FMAU is used here to demonstrate the applicability of a novel mass spectrometric method for rapid enantiomeric determination of antiviral nucleoside agents. There have been several reports on development of methods of chiral analysis based solely on mass spectrometry.[6−11] Among them, a method[9,12,13] based on competitive dissociations of transition metal ion bound complexes shows great promise in quantitative analysis of amino acids.[9,12,14] It has been applied to several pharmaceutical compounds in demonstration experiments.[15] A trimeric cluster ion, comprised of an analyte (A, the chiral compound of interest either as a pure form or a mixture of optical isomers), a chiral reference compound (ref*), and a transition metal ion (M), is generated by electrospray ionization mass spectrometry (ESI-MS). The mass-selected cluster ion is then subjected to low energy dissociation. Chiral discrimination is achieved in the dissociation of these cluster ions and evaluated by the kinetic method[16,17] for the enantiomeric quantification of chiral mixtures.

We apply this newly developed method here in the first report of gas-phase enantiomeric quantification of a drug candidate, a chiral antiviral nucleoside drug. Besides its speed and high sensitivity—characteristics of most mass spectrometric approaches—this method displays several unique features, including accurate chiral quantification without requiring isotopic labeling or wet chemical steps. The method is tolerant to matrix interference and is implemented using a commercial instrument.

Results and Discussion. The fundamental concept of the kinetic method for chiral analysis is illustrated in eq 1. The experiments are done by simply mixing the

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analyte with a transition metal salt (M) and a chiral reference compound (ref*) in aqueous methanol solution.

$$A \xrightarrow[ESI]{M(II),\ ref^*} [M^{II}(ref^*)_2(A) - H]^+ \begin{cases} \xrightarrow{k_{ref^*}} [M^{II}(ref^*)(A) - H]^+ + ref^* \\ \xrightarrow{k_A} [M^{II}(ref^*)_2 - H]^+ + A \end{cases} \quad (1)$$

Electrospraying the solution efficiently generates singly charged trimeric ions $[M^{II}(ref^*)_2(A) - H]^+$, together with other cluster ions. The trimeric cluster ion is mass-selected and collisionally excited in a quadrupole ion trap. In this tandem mass spectrometry (MS/MS) experiment, dimeric complex ions $[M^{II}(ref^*)(A) - H]^+$ and $[M^{II}(ref^*)_2 - H]^+$ are generated by competitive loss of the neutral chiral reference compound, ref*, or the analyte, A, respectively. There is a difference in energy required to generate the diastereomeric forms of the fragment ions $[M^{II}(ref^*)(A) - H]^+$, due to the two configurations of the analyte A. This results in differences in the measurable product ion abundance ratio, $[M^{II}(ref^*)(A) - H]^+$ relative to the abundance of $[M^{II}(ref^*)_2 - H]^+$. The relative branching ratio R (eq 2) is given by

$$R = [M^{II}(ref^*)(A) - H]^+/[M^{II}(ref^*)_2 - H]^+ \quad (2)$$

When the analyte is enantiomerically pure, R becomes R_D or R_L, and the chiral selectivity R_{chiral}, the ratio of R_D and R_L (eq 3), reflects the degree chiral distinction. The more different the R_{chiral} value is from unity, the higher the degree of chiral recognition.

$$R_{chiral} = \frac{R_D}{R_L} = \frac{[M^{II}(A_D)(ref^*) - H]^+/[M^{II}(ref^*)_2 - H]^+}{[M^{II}(A_L)(ref^*) - H]^+/[M^{II}(ref^*)_2 - H]^+} \quad (3)$$

Figure 1 illustrates typical product ion MS/MS spectra showing the behavior of mixtures of D- and L-FMAU in different proportions, using *N*-acetyl-L-proline as the reference and Co^{2+} as the central ion. The singly charged trimeric complex ion $[Co^{II}(ref^*)_2(A) - H]^+$ fragments simply by the loss of a neutral ligand, either reference or analyte, to form two deprotonated diastereomeric dimers. The results, shown in Figure 1, indicate that the branching ratio for these fragmentations depends strongly on the stereochemistry of the drug. When A is pure D-FMAU, the branching ratio R_D is 0.75 (Figure 1a) whereas R_L is 0.40 for pure L-FMAU (Figure 1c). When A is the racemic mixture, R becomes 0.55 (Figure 1b). The chiral selectivity, R_{chiral}, is found to be 1.88 in this case, under the activation condition chosen.

Figure 1 demonstrates that the enantiomeric excess (ee) of L-FMAU affects the R value. To quantify chiral drug mixtures, however, a calibration curve relating R and ee must be constructed. If a relatively simple relationship between R and ee can be established, then rapid determination of chiral mixtures will be possible by simply measuring the branching ratio R in a single tandem mass spectrum.

The calibration curve was constructed by measuring individual R values for a pure chiral reference and an analyte composed of FMAU samples with different ee of L-FMAU. A linear relationship of ln(R) versus ee was observed (Figure 2) with a correlation coefficient

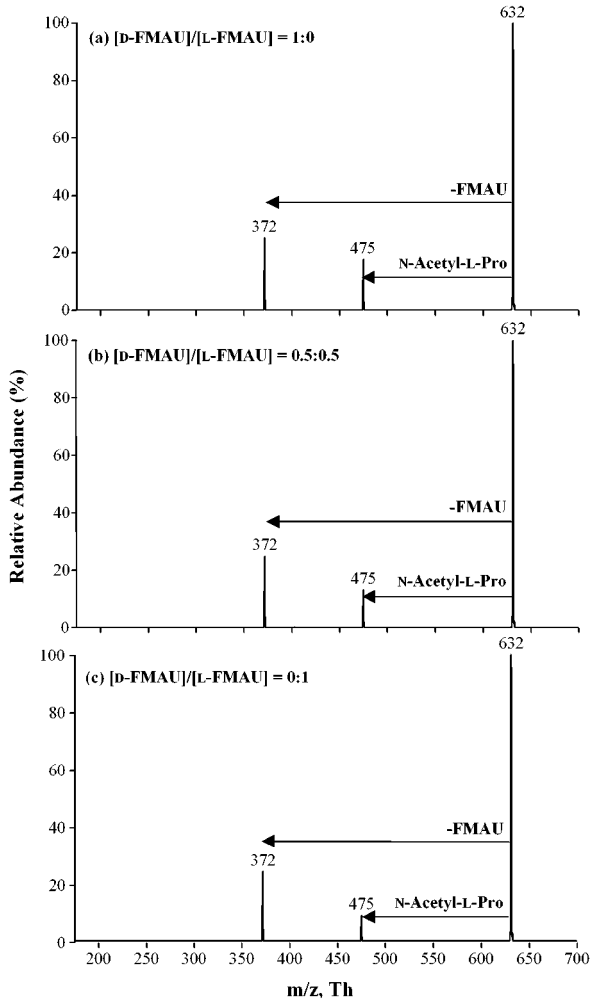

Figure 1. MS/MS product ion spectra of $[Co^{II}(N\text{-acetyl-L-Pro})_2(FMAU) - H]^+$ (m/z = 632) for mixtures of D- and L-FMAU in different proportions. The CID activation level chosen (12%) corresponds to approximately 300 mV AC excitation.

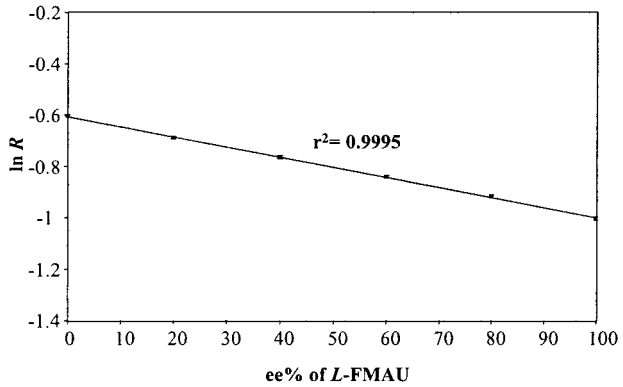

Figure 2. Calibration curve for chiral analysis of enantiomeric excess of L-FMAU in a mixture of D-FMAU and L-FMAU, using *N*-acetyl-L-Pro as the chiral reference ligand and Co(II) as the central metal ion. Each calibration point shows error bars and represents the average of three individual measurements.

(r^2) of 0.9995, using *N*-Ac-L-Pro as the ref* and Co^{2+} as the central ion. The above calibration curve was then used to measure the percent ee of "unknown" samples. The analysis of each unknown sample requires only one measurement in a single MS/MS spectrum, al-

Table 1. Influence of the Transition Metal Ion and Reference Ligand on Chiral Distinction of D- and L-FMAU[a]

	R_{chiral}			
ref*	$M = Co^{II}$	$M = Ni^{II}$	$M = Cu^{II}$	$M = Zn^{II}$
L-Ile	*b*	*b*	0.82	0.82
N-acetyl-L-Phe	1.84	1.36	1.22	1.51
N-acetyl-L-Pro	1.88	2.82	1.03	3.15
thymidine	0.81	0.84	0.87	0.97

[a] CID activation level is optimized in each experiment and then kept constant for the measurement of the isomers. [b] R_{chiral} not available due to the loss of L-Ile only from the trimeric cluster ion.

though replicates were made to increase precision. An average accuracy of 0.6%ee was obtained for this particular case.

The linear relationship of ln(*R*) versus ee is intrinsic to the kinetic method[16,17] and is the result of the logarithmic relationship between relative abundance and internal energy that characterizes unimolecular dissociation of isolated ions. A detailed derivation based on the kinetics and energetics of the competitive dissociations is given elsewhere.[9,13] Experimental data, as shown in Figure 2, confirm the existence of such a relationship for the case of D,L-FMAU. Quantitative measurements require only a two-point calibration using a racemic sample and a sample of known ee. Subsequent analysis of unknowns can be carried out by measuring the ratio of two fragment ions in a single spectrum, within a time of less than 1 min.

Because of the absence of solvent in the mass spectrometer, the method depends strongly on the choice of chiral reference compound and the strength of the metal−ligand interactions for chiral recognition and quantitative analysis. The effect of the choice of chiral reference and central ion on the ease of chiral recognition is reflected in the R_{chiral} values, summarized in Table 1. The choice of appropriate reference will be based in part on its production of large steric interactions.

Structural similarity to the analyte is also a desirable quality because it usually facilitates ready complex formation and gives similar rates of competitive dissociation that allow accurate measurements of relative abundance ratios. Thymidine has a similar structure to FMAU, but only a small chiral distinction was observed when it is used as the chiral reference. L-Isoleucine gives neither a large chiral distinction nor an appropriate branching ratio for accurate measurements. Among all reference compounds examined, *N*-acetyl-L-proline is the best choice since it gives a large chiral selectivity with Co(II), Ni(II), and Zn(II) as the central ion. The effect of the selection of different central ions is intriguing. In contrast to the case of amino acids, copper(II) as the central ion results in a very small chiral selectivity while zinc(II) and nickel(II) ions give the largest chiral recognitions. The exact binding sites in these transition metal ion bound complexes are not known but there are two binding sites in nucleosides: one the basic group and the other, which seems to be less important, is the carbohydrate moiety. The order of chiral recognition with various metal ions roughly parallels the Irving−Williams series[18] but in the opposite direction. For instance, when *N*-acetyl-L-phenylalanine is the chiral reference, chiral recognition of D/L-FMAU follows the order $Co^{2+} > Ni^{2+} > Cu^{2+} < Zn^{2+}$. Copper(II) is expected to form the most stable complexes with FMAU, but this occurs through its basic group which is remote from the chiral centers, hence the very small chiral selectivity. A complete study of chiral interactions in these cluster ions is in progress.

In conclusion, tandem mass spectrometry of transition metal ion bound complexes provides a rapid, sensitive, and accurate method for direct quantitation of this chiral antiviral agent. The measurements are carried out on a commercial instrument using standard ESI mass spectrometry, they require only very small amounts of sample for analysis, and a few percent ee can be easily determined. This study, along with the previous successful chiral analysis of other types of compounds[13,15,19] by the same approach, suggests that the kinetic method may represent a general and practical mass spectrometric method for fast chiral analysis.

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Supporting Information Available: Experimental details and MS spectrum for the formation of transition metal ion bound cluster ions as well as details of quantitative analysis of unknown samples. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Uchida, H.; Kodama, E. N.; Yoshimura, K.; Maeda, Y.; Kosalaraksa, P.; Maroun, V.; Qiu, Y.-L.; Zemlicka, J.; Mitsuya, H. In vitro anti-human immunodeficiency virus activities of Z- and E-methylenecyclopropane nucleoside analogues and their phosphoro-L-alaninate diesters. *Antimicrob. Agents Chemother.* **1999**, *43*, 1487−1490.

(2) Ono, S. K.; Kato, N.; Shiratori, Y.; Kato, J.; Goto, T.; Schinazi, R. F.; Carrilho, F. J.; Omata, M. The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. *J. Clin. Invest.* **2001**, *107*, 449−455.

(3) Han, H.-K.; De Vrueh, R. L. A.; Rhie, J. K.; Covitz, K.-M. Y.; Smith, P. L.; Lee, C.-P.; Oh, D.-M.; Sadee, W.; Amidon, G. L. 5′-Amino acid esters of antiviral nucleosides, acyclovir, and AZT are absorbed by the intestinal PEPT1 peptide transporter. *Pharm. Res.* **1998**, *15*, 1154−1159.

(4) Yao, G. Q.; Liu, S.; Chou, E.; Kukhanova, M.; Chu, C. K.; Cheng, Y. Inhibition of EBV replication by a novel L-nucleoside, 2′-fluoro-5-methyl-beta-L-arabinofuranosyluracil. *Biochem. Pharmacol.* **1996**, *51*, 941−947.

(5) Blum, M. R.; Chittick, G. E.; Wang, L. H.; Keilholz, L. J.; Fang, L.; Szczech, G. M.; Rousseau, F. S. Clevudine (L-FMAU) a new agent under development for the treatment of hepatitis B virus (HBV): Evaluation of the safety, pharmacokinetics and effect of food following single-dose administration in healthy male volunteers. *Hepatology* **2000**, *32*, 1702−1706.

(6) Filippi, A.; Giardini, A.; Piccirillo, S.; Speranza, M. Ga-phase enantioselectivity. *Int. J. Mass Spectrom.* **2000**, *198*, 137−163.

(7) Sawada, M.; Takai, Y.; Yamada, H.; Nishida, J.; Kaneda, T.; Arakawa, R.; Okamoto, M.; Hirose, K.; Tanaka, T.; Naemura, K. Chiral amino acid recognition detected by electrospray ionization (ESI) and fast atom bombardment (FAB) mass spectrometry (MS) coupled with the enantiomer-labelled (EL) guest method. *J. Chem. Soc., Perkin Trans. 2* **1998**, *3*, 701−710.

(8) Guo, J.; Wu, J.; Siuzdak, G.; Finn, M. G. Measurement of enantiomeric excess by kinetic resolution and mass spectrometry. *Angew. Chem., Int. Ed.* **1999**, *38*, 1755−1758.

(9) Tao, W. A.; Zhang, D.; Nikolaev, E. N.; Cooks, R. G. Copper(II)-assisted enantiomeric analysis of D,L-amino acids using the kinetic method: chiral recognition and quantification in the gas phase. *J. Am. Chem. Soc.* **2000**, *122*, 10598−10609.

(10) Grigorean, G.; Ramirez, J.; Ahn, S. H.; Lebrilla, C. B. A mass spectrometry method for the determination of enantiomeric

excess in mixtures of D,L-amino acids. *Anal. Chem.* **2000**, *72*, 4275−4281.
(11) Smith, G.; Leary, J. A. Differentiation of diastereomeric nickel-(II) N-glycoside complexes using tandem mass spectrometry and kinetic energy release measurements. *J. Am. Chem. Soc.* **1996**, *118*, 3293−3294.
(12) Tao, W. A.; Zhang, D.; Wang, F.; Thomas, P.; Cooks, R. G. Kinetic resolution of D, L-amino acids based on gas-phase dissociation of copper(II) complexes. *Anal. Chem.* **1999**, *71*, 4427−4429.
(13) Tao, W. A.; Cooks, R. G. Parallel reactions for enantiomeric quantification of peptides by mass spectrometry. *Angew. Chem., Int. Ed.* **2001**, *40*, 757−760.
(14) Zhang, D.; Tao, W. A.; Cooks, R. G. Chiral resolution of D- and L-amino acids by tandem mass spectrometry of Ni(II)-bound trimeric complexes. *Int. J. Mass Spectrom.* **2001**, *204*, 159−169.
(15) Tao, W. A.; Gozzo, F. C.; Cooks, R. G. Mass spectrometric quantitation of chiral drugs by the kinetic method. *Anal. Chem.* **2001**, *73*, 1692−1698.
(16) Cooks, R. G.; Patrick, J. S.; Kotiaho, T.; McLuckey, S. A. Thermochemical determinations by the kinetic method. *Mass Spectrom. Rev.* **1994**, *13*, 287−339.
(17) Cooks, R. G.; Wong, P. S. H. Kinetic method of making thermochemical determinations. *Acc. Chem. Res.* **1998**, *31*, 379−386.
(18) Irving, H.; Williams, R. J. P. Order of stability of metal complexes. *Nature* **1948**, *162*, 746−747.
(19) Tao, W. A.; Wu, L.; Cooks, R. G. Rapid enantiomeric determination of α-hydroxy acids by electrospray ionization tandem mass spectrometry. *Chem. Commun.* **2000**, *20*, 2023−2024.

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