Chromatographic and Mass Spectral Methods of Identification for the Side-Chain and Ring Regioisomers of Methylenedioxymethamphetamine

Laura Aalberg[1,2], **Jack DeRuiter**[1], **F. Taylor Noggle**[3], **Erkki Sippola**[2], and **C. Randall Clark**[1,*]

[1]Department of Pharmacal Sciences, School of Pharmacy, Auburn University, Auburn, AL 36849; [2]National Bureau of Investigation, Crime Laboratory, 01300 Vantaa, Finland; and [3]Alabama Department of Forensic Sciences, Wire Road, Auburn, AL 36830

Abstract

The popular drug of abuse 3,4-methylenedioxymethamphetamine (MDMA) is one of a total of 10 regioisomeric 2,3- and 3,4-methylenedioxyphenethylamines of MW 193 that yields regioisomeric fragment ions with equivalent mass (m/z 58 and 135/136) in the electron-impact (EI) mass spectrum. Thus, these 10 methylenedioxyphenethylamines are uniquely isomeric; they have the same molecular weight and equivalent major fragments in their mass spectra. The specific identification of one of these compounds (i.e., Ecstasy or 3,4-MDMA) in a forensic drug sample depends upon the analyst's ability to eliminate the other regioisomers as possible interfering or coeluting substances. This study reports the synthesis, chemical properties, spectral characterization, and chromatographic analysis of these 10 unique regioisomers. The ten 2,3- and 3,4-regioisomers of MDMA are synthesized from commercially available precursor chemicals. In the EI mass spectra, the side-chain regioisomers show some variation in the relative intensity of the major ions, with the exception of only one or two minor ions that might be considered side-chain specific fragments. The position of substitution for the methylenedioxy ring is not easily determined by mass spectral techniques, and the ultimate identification of any one of these amines with the elimination of the other nine must depend heavily upon chromatographic methods. The chromatographic separation of these 10 uniquely regioisomeric amines are studied using reversed-phase liquid chromatographic methods with gradient elution and gas chromatographic techniques with temperature program optimization.

Introduction

The ability to distinguish between regioisomers directly enhances the specificity of the analysis for the target drugs of abuse (1–4). The mass spectrum is often the confirmatory piece of evidence for the identification of drugs of abuse in the forensic laboratory. Although the mass spectrum is often considered a specific "fingerprint" for an individual compound, there may be other substances capable of producing very similar or almost identical mass spectra. For major drugs of abuse such as the amphetamines (2,3,5) and methylenedioxymethamphetamines (MDMAs) (1,4,6), there may be many positional isomers (regio-isomers) in the alkyl side-chain or the aromatic ring substitution pattern that yield the same mass spectrum. For example, methamphetamine and phentermine (3) are regioisomeric based on the substitution of a methyl group on the nitrogen or carbon of the side chain. The street drug 4-bromo-2,5-dimethoxy- phenethylamine (known as Nexus) also has several regioisomers based on other aromatic ring substitution patterns (7). Although nuclear magnetic resonance (NMR) can be a very useful method for regioisomer differentiation, it is not a technique that has direct application for all areas of forensic drug chemistry, and it is not readily available in most forensic laboratories. Thus, the analysis of street drug samples and analytical toxicology must depend heavily upon chromatographic methods as well as mass spectrometry (MS).

When other compounds exist that have the potential to produce the same or nearly identical mass spectrum as the drug of interest, the identification by gas chromatography (GC)–MS must be based primarily upon the ability of the chromatographic system to separate the counterfeit substance from the actual drug of abuse. Those substances coeluting with the drug of abuse in chromatographic systems could be misidentified as the drug of abuse. Without the appropriate standards, a thorough method validation is not possible, thus the coelution of drug and nondrug combinations would remain a possibility. The ultimate question then is that if a forensic scientist has never analyzed all of the regioisomeric substances, how can they be sure that these compounds would not coelute with the drug of abuse. The significance of this question is related to many factors, the most important of these being the separation power of the chromatographic system and the number of possible counterfeit sub-

* Author to whom correspondence should be addressed.

stances. Furthermore, the ability to distinguish between these regioisomers directly enhances the specificity of the analysis for the target drugs of abuse.

There are 9 other methylenedioxy-substituted phenethyl-amines with the potential to produce a mass spectrum that is essentially the same as 3,4-MDMA (Figure 1), and the precursor substances exist to prepare all 9 of these counterfeit molecules. Many of these compounds are pharmacologically inactive and others have unknown pharmacological properties, yet all have the strong possibility to be identified as 3,4-MDMA by several analytical methods. In this study, all 10 ring and side-chain regioisomers of 3,4-MDMA (shown in Figure 1) were prepared and compared by chromatographic and spectroscopic techniques, and methods for their differentiation were explored with the emphasis of exploiting routine techniques used in drug-control laboratories.

Because this was the first time that all 10 of these isomeric compounds have been reported together, a direct comparison of their critical piece of forensic analytical data (the mass spectra) was very important. The actual spectra have been included to best illustrate this comparison.

Experimental

Instruments

GC–MS analyses were performed with an HP 6890 GC coupled with an HP 5973 mass selective detector (Hewlett-Packard, Little Falls, DE). The MS was operated in the electron-impact (EI) mode using an ionization voltage of 70 eV and a source temperature of 230°C. The samples were dissolved in a TRIS buffer (1 mg/mL) (pH 8.9), extracted with iso-octane (1 mL), and introduced (1.0 µL) into the MS via the GC equipped with an HP 7673 automatic injector. The separation was carried out on a 25-m × 0.20-mm-i.d. column coated with 0.33 µm of dimethyl silicone (Ultra-1), which was purchased from Hewlett-Packard. The column temperature
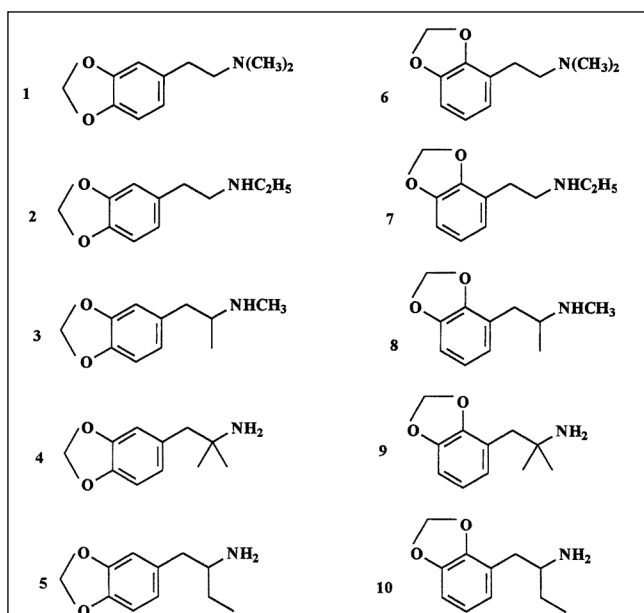
was held at 90°C for 1.0 min and then programmed to 300°C at a rate of 10°C/min with a hold time of 5 min. The samples were introduced in the splitless mode at 260°C. The split purge valve was opened after 1 min. The helium carrier gas was adjusted to 25 cm/s at 90°C in the constant flow mode.

The temperature program optimization was carried out using Drylab GC software (LC Resources Inc., Lafayette, CA). The system enables the prediction of separation with any temperature program after 2 initial runs that used different linear temperature programs have been performed.

Liquid chromatographic (LC) analyses were conducted using an HP 1090 high-performance liquid chromatograph (HPLC) with a diode array detector. The analytical column had a 15-cm × 4.6-mm-i.d., dp 5-µm Hypersil Hypurity Elite C_{18} with an analogous precolumn (10- × 4.6-mm i.d.), which was purchased from Shandon HPLC (Cheshire, UK). The mobile phase consisted of phosphate buffer (pH 3.0) and acetonitrile, which was pumped with a flow rate of 1 mL/min. The phosphate buffer was prepared by mixing 9.2 g of monobasic sodium phosphate in 1 L of double-distilled water and adjusting the pH to 3.0 with H_3PO_4. The samples were prepared in the phosphate buffer and volumes of 10 µL injected. The diode array detector recorded an ultraviolet (UV) spectra of 220 to 400 nm and a fixed wavelength of 280 nm.

Synthesis of the regioisomers
Synthesis of the 3,4-methylenedioxy compounds

The 3,4-MDMA (compound 3 in Figure 1) and butanamine (compound 5 in Figure 1) were prepared by reductive amination of 3,4-methylenedioxy- phenyl-2-propanone and 3,4-methylene-dioxy- phenyl-2-butanone with the appropriate amines as

Figure 1. Structures of the 2,3- and 3,4-methylenedioxyphenalkylamine regioisomers in this study (MW = 193, *m/z* 58 fragment ions).
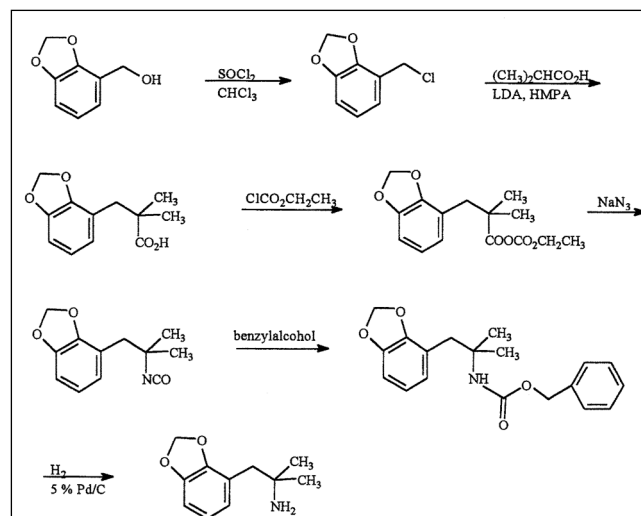

Figure 2. Synthesis of 2,3-methylenedioxyphenyl-2,2-dimethylethanamine.
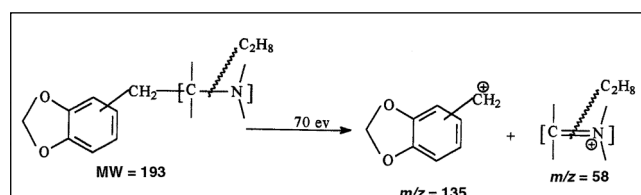

Figure 3. General mass spectral fragmentation for the methylenedioxyphenalkylamine regioisomers in this study.
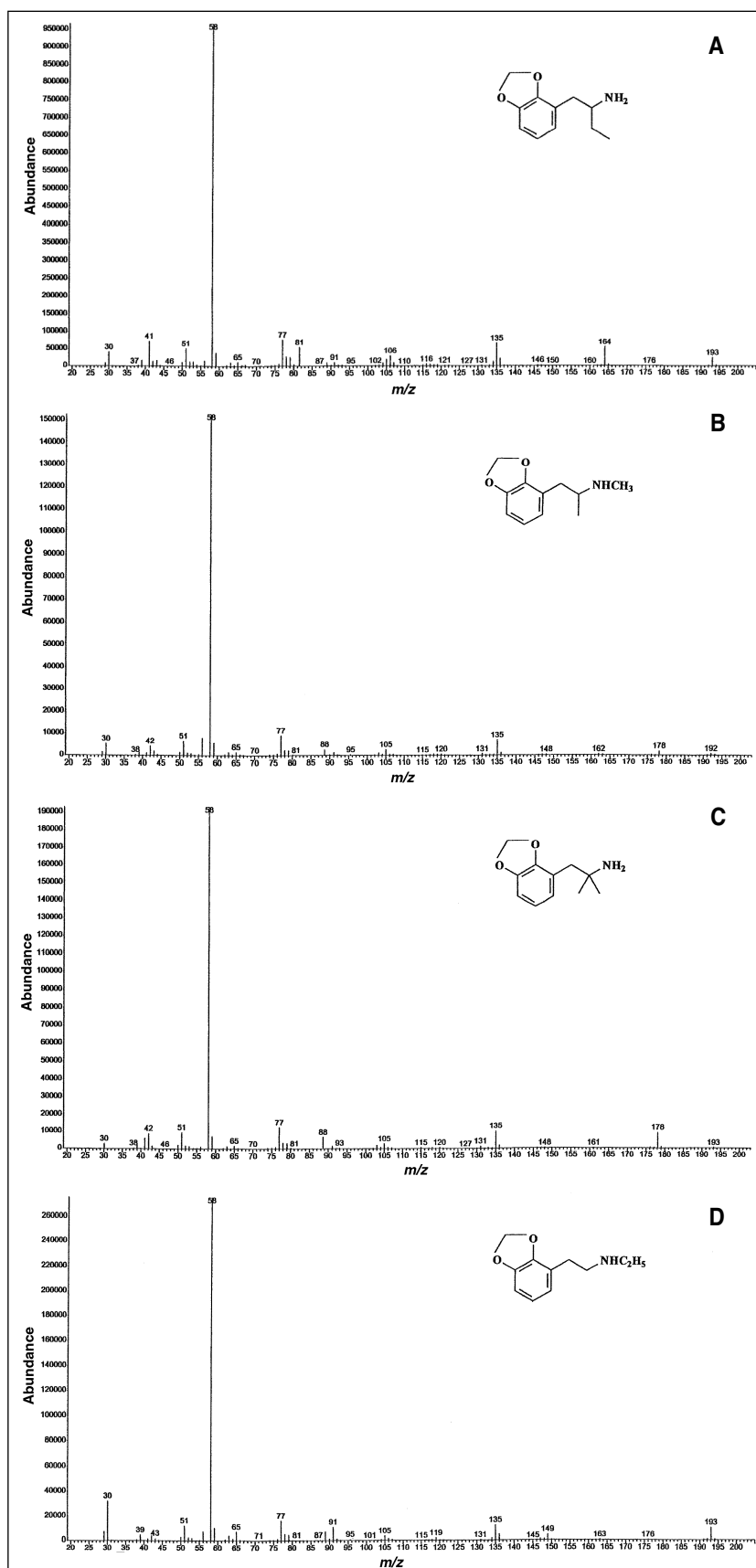
Figure 4. Mass spectrum for the 2,3-methylenedioxyphenyl-2-butanamine (A), the 2,3-methylene-dioxymethamphetamine (B), the 2,2-dimethyl-1-(2,3-methylenedioxyphenyl)-2-ethanamine (C), the N-ethyl-1-(2,3-methylenedioxyphenyl)-2-ethanamine (D), and the N,N-dimethyl-1-(2,3-methylene-dioxyphenyl)-2-ethanamine (E).

The 2,3-MDMA (compound 8 in Figure 1) was prepared from 2,3-dihydroxybenzaldehyde using the method of Casale et al. (10). The butanamine (compound 10 in Figure 1) was prepared from 2,3-dihydroxybenzaldehyde via the 2,3-methyl-enedioxyphenyl-2-nitrobutene intermediate (1). The N-alkyl-2,3-methylenedioxyphenethyl-amines (compounds 6 and 7 in Figure 1) were prepared by the acylation of the amine, followed by lithium aluminum hydride reduction.

Synthesis of the 2,3- and 3,4-methylene-dioxyphenyl-2,2-dimethylethanamine

The same method was used to prepare the 2,3- and 3,4-regioisomers; therefore, only one example will be described. A solution of 2,3-methylenedioxybenzaldehyde in 2-propanol was reduced to the benzyl alcohol using excess sodium borohydride. The alcohol was converted to the benzyl chloride using thionyl chloride in refluxing chloroform and isolated by Kugelrohr distillation.

A solution of di-isopropylamine in tetrahydro-furan under a dry nitrogen atmosphere was cooled with external dry ice in 2-propanol, followed by the addition of a solution of n-butyl-lithium in hexane. Isobutyric acid was added dropwise to the resulting mixture along with hexamethylphospho-ramide. Upon warming to room temperature, the 2,3-methylenedioxybenzyl chloride was added and the mixture was stirred overnight at room temper-ature. 2,2-Dimethyl-(2,3-methylenedioxyphenyl)-propionic acid was isolated as a yellow crystalline solid.

2,2-Dimethyl-(2,3-methylenedioxyphenyl)p-ropionic acid was allowed to react with ethyl chloroformate in acetone, followed by the addi-tion of a solution of sodium azide in water. A toluene solution of the resulting azide was heated (100°C) until nitrogen evolution ceased, the sol-vent was removed under vacuum, and the residue was dissolved in benzyl alcohol. This solution was heated, followed by removal of the excess benzyl alcohol to yield a residue of 1-N-(benzyloxycar-bonyl)amino-1,1-dimethyl-2-(2,3-methylene-dioxyphenyl)ethane as an amber oil.

The oily residue was dissolved in ethanol and hydrogenated over 10% palladium on carbon. Treatment of the resulting residue with anhy-drous HCl gave the desired amine as a white crys-talline solid.

Results and Discussion

Preparation of the regioisomers

The ten 2,3- and 3,4-regioisomers of MDMA, N-

ethyl-methylenedioxyphenethylamine, 1-methylenedioxyphenyl-2-butanamine (known as BDB), *N,N*-dimethyl-1-methylene-dioxy- phenyl-2-ethanamine, and 1-methylenedioxy-phenyl-2,2-dimethylethanamine were synthesized from commercially available precursor chemicals.

The methods for the preparation of many of the 2,3- and 3,4-

Figure 4. (continued) Mass spectrum for the 2,3-methylenedioxyphenyl-2-butanamine (A), the 2,3-methylenedioxymethamphetamine (B), the 2,2-dimethyl-1-(2,3-methylenedioxyphenyl)-2-ethanamine (C), the *N*-ethyl-1-(2,3-methylenedioxyphenyl)-2-ethanamine (D), and the *N,N*-dimethyl-1-(2,3-methylenedioxyphenyl)-2-ethanamine (E).

Figure 5. Mass spectrum for the 3,4-methylenedioxyphenyl-2-butanamine (A), the 3,4-methylene-dioxymethamphetamine (B), the 2,2-dimethyl-1-(3,4-methylenedioxyphenyl)-2-ethanamine (C), the *N*-ethyl-1-(3,4-methylenedioxyphenyl)-2-ethanamine (D), and the *N,N*-dimethyl-1-(3,4-methylene-dioxyphenyl)-2-ethanamine (E).

methylenedioxy-regioisomers have been described in previous reports (1,6,8,9). The general procedure for the synthesis of these compounds uses 2,3- and 3,4-methylenedioxybenzaldehyde (piperonal) as starting materials. The preparation of 2,3-methylenedioxybenzaldehyde has been reported previously (1,10). Condensation of the appropriate aldehyde with a nitroalkane (nitromethane, nitroethane, or 1-nitropropane) under basic conditions yielded the 2-nitroalkenes in which reduction with lithium aluminum hydride (LAH) yielded the primary amines. The *N*-methyl and *N*-ethyl analogs were prepared from the primary amines by acylation, followed by LAH reduction. The 1-(2,3- and 3,4-methyl- ene-dioxyphenyl)-2,2-dimethylethan-amines were prepared from the aldehydes according to the methods outlined in Figure 2. The aldehydes were reduced to the alcohol and converted to the benzyl chloride that was condensed with isobutyric acid using butyl lithium to yield 2,2-dimethyl-3-(2,3- and 3,4-methylenedioxyphenyl)-1-propionic acid. The acid was treated sequentially with sodium azide, ethyl chloroformate, and benzyl alcohol, followed by catalytic hydrogenation under low pressure to yield the desired 1-(2,3- and 3,4-methylenedioxyphenyl)-2,2-dimethylethanamines.
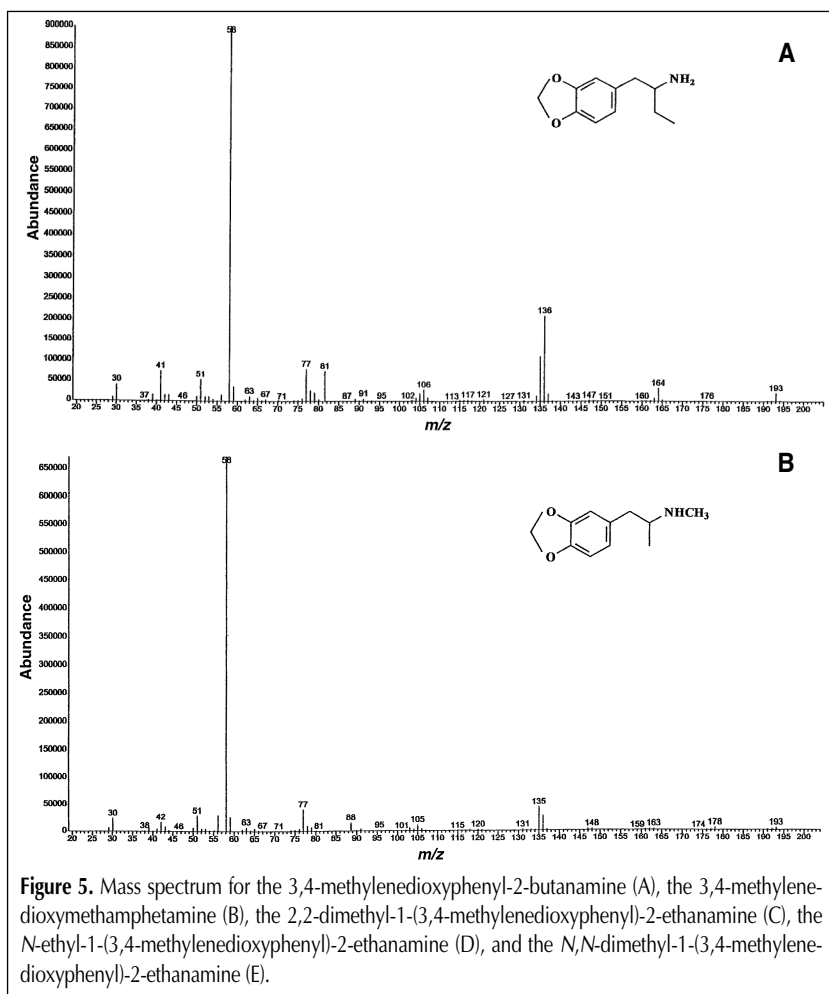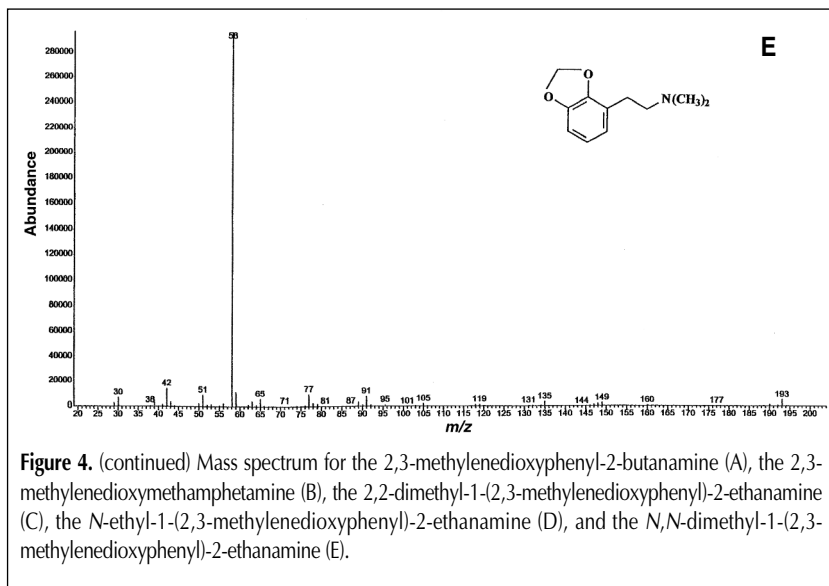
Mass spectrometry

MS is the primary method used for confirming the identity of drugs and other substances of abuse in forensic samples. The mass spectra of phenethylamine drugs of abuse (including 3,4-MDMA) are characterized by a base peak formed by an alpha-cleavage reaction involving the carbon–carbon bond of the ethyl linkage between the aromatic ring and the amine. In 3,4-MDMA (MW = 193), the alpha-cleavage reaction yields the 3,4-methylenedioxybenzyl fragment at mass 135/136 (for the cation and the radical cation, respectively) and the substituted imine fragment at *m/z* 58. Thus, the mass spectrum for 3,4-MDMA contains major ions at *m/z* 58 and 135/136 as well as other ions of low relative abundance.

There are 9 regioisomers (a total of 10 compounds) of the 3,4-MDMA molecule (MW = 193) that should yield alpha-cleavage fragments at *m/z* 58 and 135/136 during analysis by MS. The 10 possible ring and side-chain regio-isomers, (including 3,4-MDMA) are shown in Figure 1. The EI fragmentation reaction and the general structures for the regioisomeric product ions are shown in Figure 3. Although there are many other regioisomeric arrangements of the 3,4-MDMA molecule, all others yield imine and benzyl fragments from the major alpha-cleavage reaction at masses other than *m/z* 58 and 135/136.

The EI mass spectra for the regioisomeric sub-

stances evaluated in this study are shown in Figures 4 and 5. The mass spectra for some of these compounds have appeared in other recent publications (1,9); however, their inclusion in this study was necessary to illustrate the similarity of these unique regioisomers. Figure 4 shows the 5 side-chain regioisomers substituted in a "2,3- manner" to the methylenedioxyphenyl ring. All compounds showed the m/z 58 ion as the base peak as well as the expected substituted benzyl fragment at m/z 135 and the molecular ion at m/z 193. The butanamine in Figure 4A also showed a fragment ion at m/z 164 from the loss of 29 mass units. This ion resulted from the loss of the ethyl-group in the less-favored alpha-clevage reaction. 2,3-MDMA (Figure 4B) showed essentially the same major fragment ions with an additional low intensity ion at

m/z 178 (M-CH$_3$). The mass spectrum for 2,2-dimethyl-ethanamine in Figure 4C was strikingly similar to that of the MDMA regioisomer in Figure 4B, showing all the expected ions in similar relative intensities. Perhaps the only difference in these two spectra was the higher relative abundance of the m/z 178 ion in the spectrum for the 2,2-dimethyl regioisomer. There are two equivalent methyl-groups that could be lost from the 2,2-dimethyl regioisomer (Figure 4C) in an alpha-cleavage reaction; this likely accounted for the greater intensity of the m/z 178 fragment in Figure 4C. The other two spectra in Figure 4 (4D and 4E) for the N-ethyl and N,N-dimethylethanamine regioisomers did not show any high mass fragments other than the molecular ion at m/z 193. However, these spectra were quite similar to those of the other regioisomers in Figure 4. As expected, there were very few specific ions of significant relative intensity that were useful for the specific identification of any of these regioisomeric substances.

The mass spectra in Figure 5 were for the 5 side-chain regioisomers of the 3,4-methylenedioxyphenyl substitution pattern. In a direct comparison with the 2,3-regioisomers of the identical side chain, the major difference was the greater relative abundance of the radical cation at m/z 136 for the 3,4-substitution pattern in most cases. The lower intensity of the radical cation species for the 2,3-methylenedioxyphenyl-regioisomers was perhaps the result of steric impediments to this hydrogen-transfer rearrangement. The radical cation species at m/z 136 was a significant fragment in the spectrum for 3,4-methylenedioxyphenyl-2-butanamine (shown in Figure 5A). The spectra for 3,4-MDMA and 2,2-dimethylethanamine (Figure 5B and 5C, respectively) were virtually identical as observed for the 2,3-regioisomers. The spectrum in Figure 5D again showed a significant amount of the m/z 136 ion for N-ethyl-1-(3,4-methylenedioxyphenyl)-2-ethanamine, and the N,N-dimethyl-regioisomer did not indicate any detectable amount of the 136 ion.

GC–MS

The 10 regioisomeric amines were separated by capillary GC using a standard screening method, followed by a temperature program optimization technique. The screening method was a methyl silicone capillary column using a temperature program rate of 10°C/min. The screening method did not separate all compounds (the results are illustrated in Figure 6). Compounds 3 and 7 (3,4-MDMA and N-ethyl-2,3-methylenedioxyphenyl-2-ethanamine) completely coeluted under these conditions (see Figures 4D and 5B for a direct comparison of the mass spectra of these two coeluting regioisomers). Because compound 3 was 3,4-MDMA, this chromatographic system would
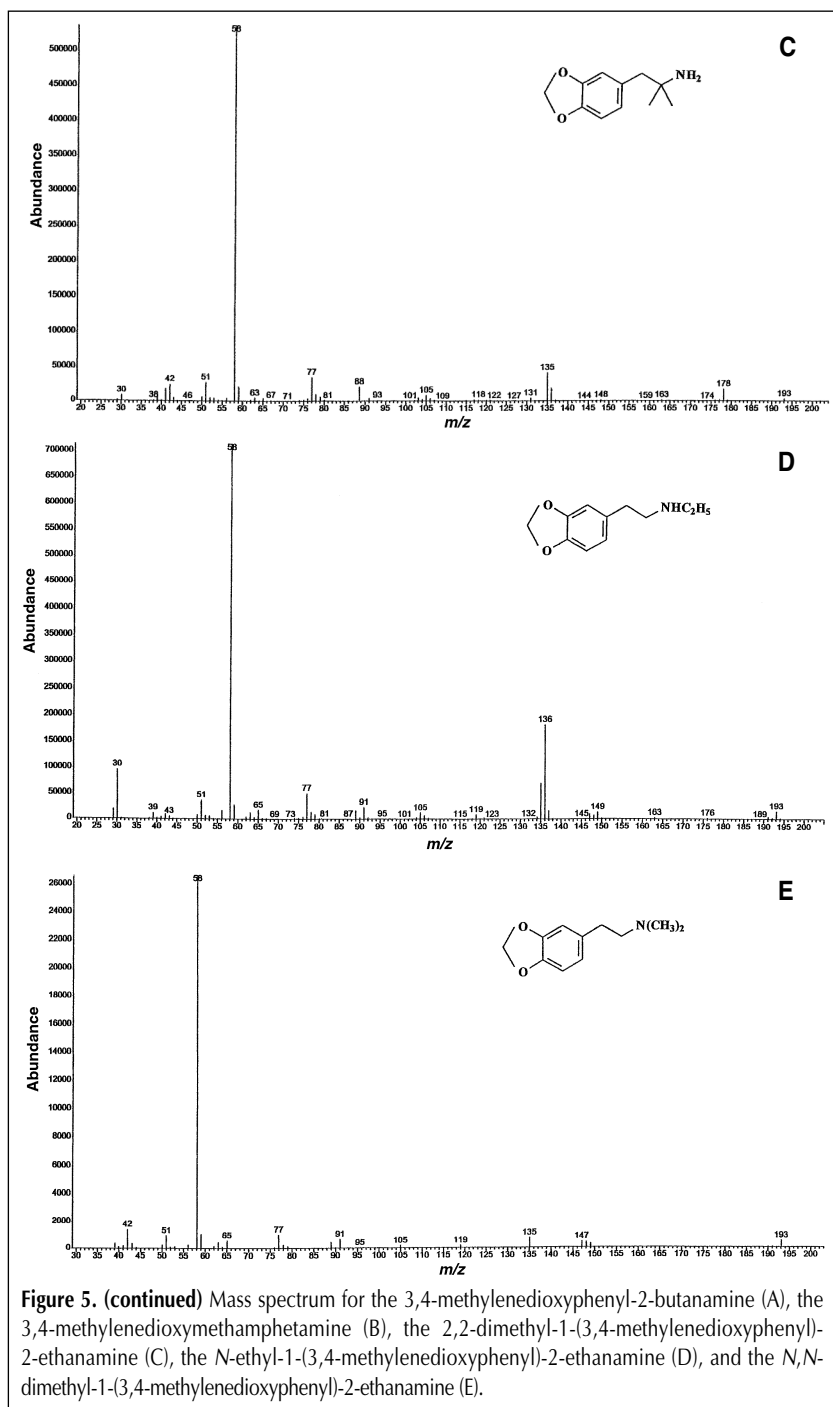

Figure 5. (continued) Mass spectrum for the 3,4-methylenedioxyphenyl-2-butanamine (A), the 3,4-methylenedioxymethamphetamine (B), the 2,2-dimethyl-1-(3,4-methylenedioxyphenyl)-2-ethanamine (C), the N-ethyl-1-(3,4-methylenedioxyphenyl)-2-ethanamine (D), and the N,N-dimethyl-1-(3,4-methylenedioxyphenyl)-2-ethanamine (E).

not be adequate for the specific identification of this drug of abuse.

In order to overcome the problem of coelution with 3,4-MDMA, a computer-based temperature program optimization technique was applied. Initially 2 runs with program rates of 2.0 and 12°C/min were performed. Column efficiency, dead time, and retention times of the analytes were entered in the Drylab GC program. The Drylab analysis revealed the existence of 2 resolution
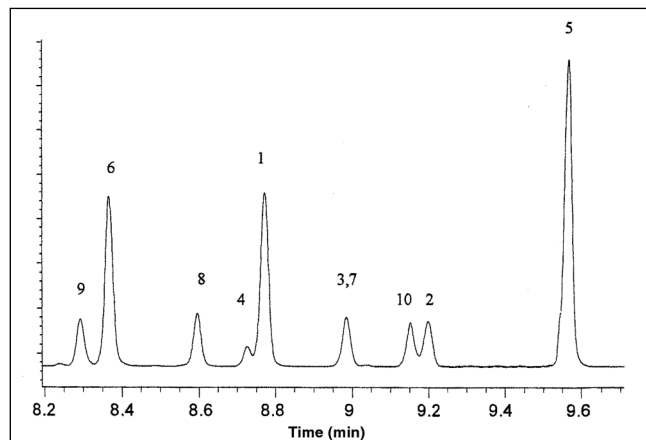
maxima (Figure 7). The highest (obtained at very low program rates) provided the best separation power with the currently used stationary phase. For reasons of practicality and analysis time, 2.0°C/min was chosen as the optimized program rate, although

Figure 6. The chromatogram obtained from the GC screening method for the methylenedioxyphenalkylamines. The numbers over the peaks correspond to the compound numbers in Figure 1.
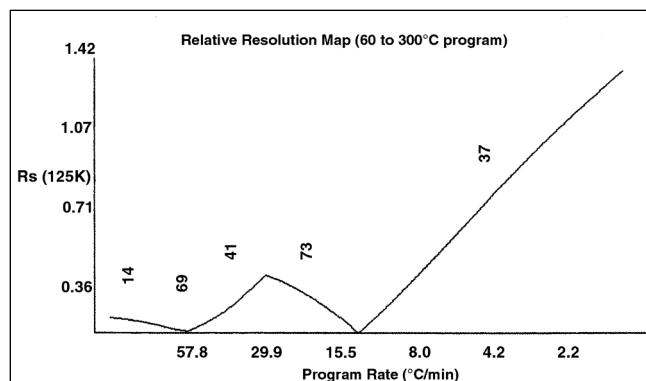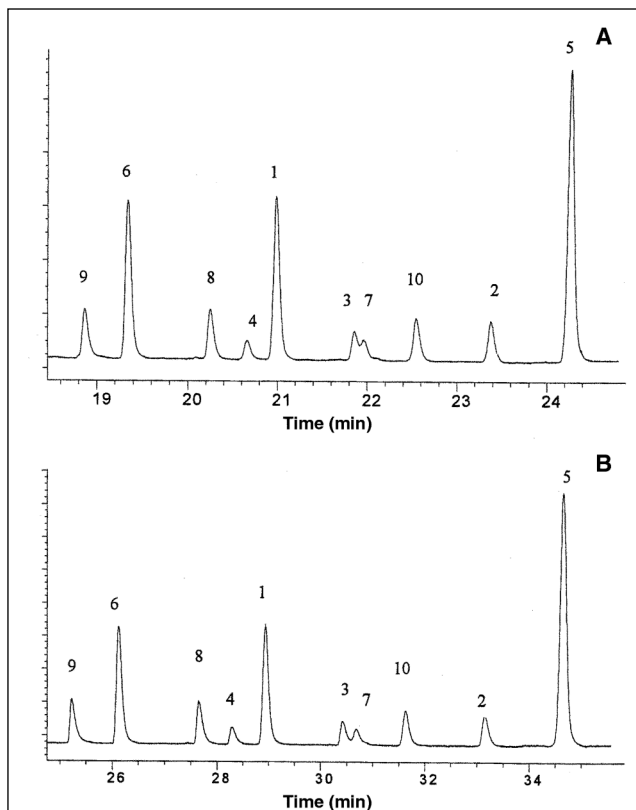

Figure 7. Relative resolution map. The maximum point on the curve indicates the best overall resolution. It should be noted that the programming rate axis (*x*-axis) is logarithmic.

Table I. Drylab Prediction for Minimum Resolution of the Critical Peak Pair as a Function of the Temperature Program Rate

Program rate	Minimum R_S	Critical peak pair	t_R of last peak (min)
1.0	1.48	3, 7	56.49
2.0	1.11	3, 7	38.81
3.0	0.87	3, 7	30.36
4.0	0.69	3, 7	25.27
5.0	0.54	3, 7	21.81
6.0	0.42	3, 7	19.30
7.0	0.32	3, 7	17.37
8.0	0.24	3, 7	15.84
9.0	0.16	3, 7	14.60
10.0	0.10	3, 7	13.56

Figure 8. GC separation of the methylenedioxyphenalkylamines using 4°C/min (A) and 2°C/min (B) program rates. The numbers over the peaks correspond to the compound numbers in Figure 1.
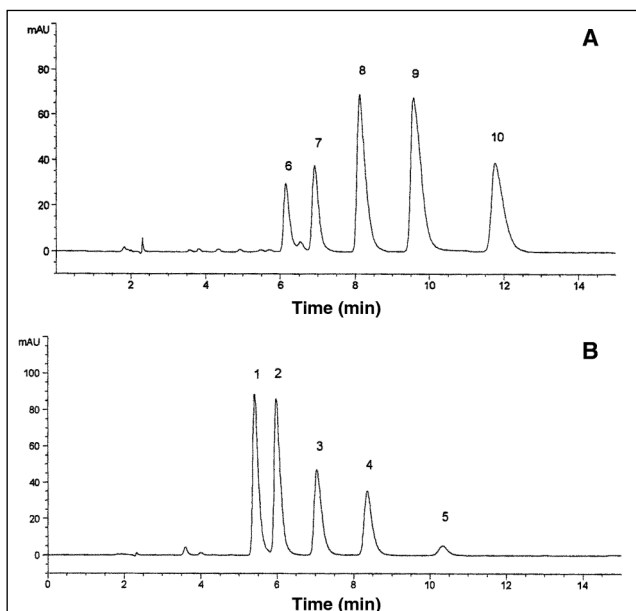

Figure 9. Liquid chromatographic separation of 2,3-regioisomers (A) and 3,4-regioisomers (B). The mobile phase consisted of 13% acetonitrile and a phosphate buffer (pH 3). The numbers over the peaks correspond to the compound numbers in Figure 1.

ships for the HPLC separation were quite different from those observed in the GC–MS analysis of these compounds. The isocratic separations in Figure 9 showed the same elution order for the side-chain isomers regardless of the position of substitution for the methylenedioxy group to the aromatic ring. The elution order in both chromatograms showed that retention increases with the chain length of the uninterrupted hydrocarbon that is attached directly to the aromatic ring. Thus, the first compounds to elute were the *N*,*N*-dimethyl- and *N*-ethyl-methylene-dioxyphenethylamines; these compounds had the least number of carbons attached directly to the aromatic ring. These compounds were followed by the MDMA derivative having a C_3 chain attached directly to the ring, and the compounds showing the highest retention had a C_4 chain attached to the aromatic ring. Additionally, the unbranched C_4 chain had a greater affinity for the hydrocarbon stationary phase than the branched arrangement of the C_4 chain. This elution order has been observed for the analogous series of side-chain regioisomeric phenalkylamines having an unsubstituted aromatic ring (methamphetamine regioisomers) on hydrocarbon stationary phases. Also, comparison of the chromatograms in Figure 9 suggests that each 2,3-regioisomer has greater retention for the stationary phase than the 3,4-regioisomer of the same side-chain structure.
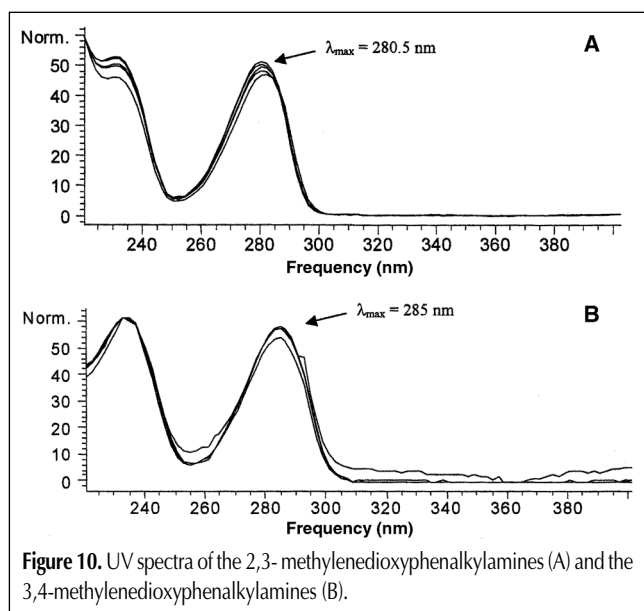

Figure 10. UV spectra of the 2,3- methylenedioxyphenalkylamines (A) and the 3,4-methylenedioxyphenalkylamines (B).
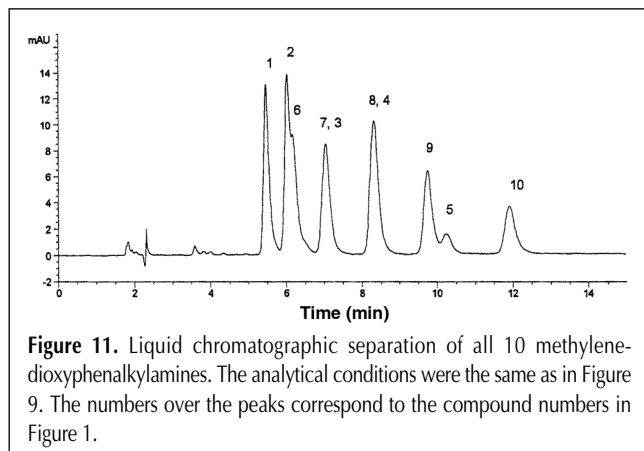

Figure 11. Liquid chromatographic separation of all 10 methylene-dioxyphenalkylamines. The analytical conditions were the same as in Figure 9. The numbers over the peaks correspond to the compound numbers in Figure 1.

The chromatogram in Figure 11 represented the initial attempt to separate all 10 regioisomers under the same chromatographic conditions used for the separations in Figure 9. This chromatogram confirmed that the 3,4-regioisomer eluted before the 2,3-regioisomer having the identical side chain. Because of the hydrophobicity difference observed for 2,3- versus 3,4-regioisomerism, some of the earlier eluting compounds coeluted (Figure 11). This is most significant when one of the coeluting compounds is 3,4-MDMA (observed in Figure 11). The coelution in this example suggested the possibility of a less than specific method for the analysis of the drug of abuse 3,4-MDMA. Without the availability of reference standards for chromatographic validation studies, the possibility of misidentification could not be eliminated.

Isocratic separation using the mixture of organic solvent, acetonitrile, methanol or tetrahydrofuran, and a phosphate buffer (pH 3) as mobile phase was studied, and the plots of compound retention (log k') versus mobile phase strength (percentage of organic solvent) are shown in Figure 12. The elution order of 2,3- and 3,4-regioisomers were the same with all 3 binary solvent systems. Compounds 3 and 7 (3,4-MDMA and 2,3-*N*-ethyl-regioisomer, respectively) were separated when the mobile phase strength was decreased; however, the elution times for the later eluting compounds were inconveniently long. The separation of 3,4-*N*-ethyl-regioisomer and 2,3-*N*,*N*-dimethyl-regioisomer was not properly achieved under any isocratic conditions on this column. The influence of pH was studied using pH values of 2.5, 3.0, 3.5, and 4.5, but such modifications of the mobile phase did not offer any improvement in the resolution of the overlapping peaks.

Gradient elution techniques allowed for an improved resolution between compounds 3 and 7. The chromatogram in Figure 13 shows the results of a reversed-phase gradient separation that used a mobile phase containing 5% acetonitrile and the phosphate buffer over 20 min and then it increased to 30% from 20 to 40 min. Although this procedure did yield adequate resolution between compounds 3 and 7, the complete resolution of all 10 compounds was not obtained on this column.

Conclusion

There are 10 compounds (3,4-MDMA and 9 other regioisomeric methylenedioxyphenalkylamines) that have molecular weights of 193 and yield regioisomeric fragment ions of equal mass in the MS. Thus, direct analysis of these compounds by EI MS does not provide data for the specific identification of one of these regioisomers to the exclusion of all the other isomers.

The separation of this set of unique regioisomers represents a considerable chromatographic challenge. The GC separation of these 10 compounds was achieved in a 2°C/min temperature program on methyl silicone requiring a total analysis time of approximately 35 min. The five 2,3-regioisomers and the five 3,4-regioisomers were separated by isocratic reversed-phase LC; however, a mixture of all 10 compounds was not completely resolved on a Hypersil C_{18} stationary phase using gradient elution. It is interesting that even with the quite different elution

orders observed in GC and HPLC, compounds 3 and 7 continued to display very similar elution properties and mass spectra. This similarity in retention properties was made more significant because compound 3 was 3,4-MDMA. Thus, under commonly used conditions for both HPLC and GC separations, these two compounds displayed very similar retention properties. The separation of *N*-ethyl-2,3-methylenedioxyphenethylamine and 3,4-MDMA may be an excellent method for the initial evaluation of
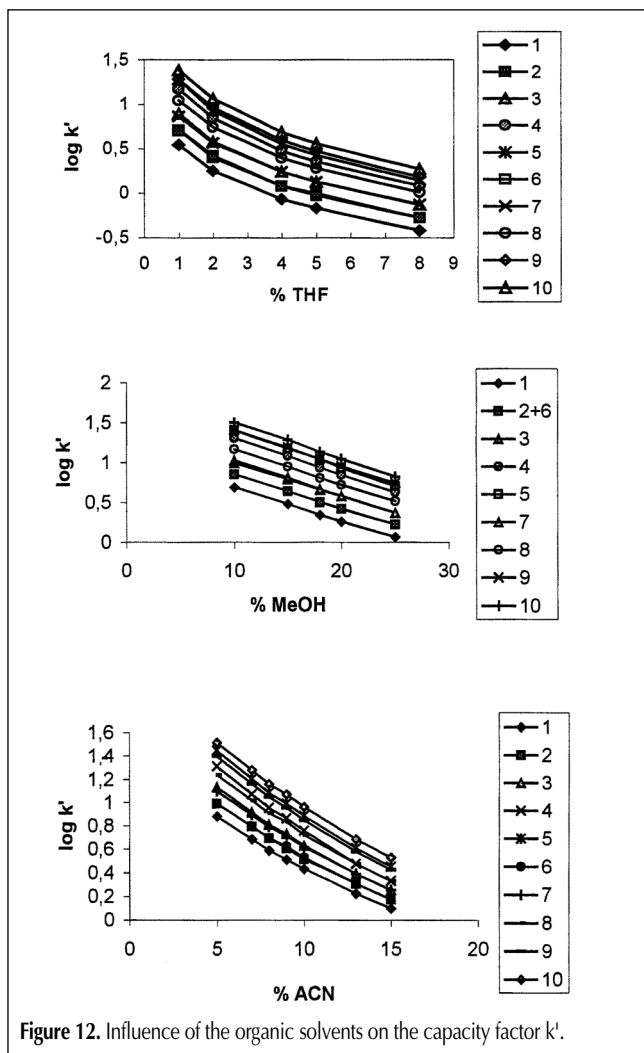
chromatographic systems used for the specific identification of drugs of this type.

It is basic wisdom that GC separation is most easily optimized by changing the nature of the stationary phase. However, the purpose of this initial study was to publish the chemical properties and some synthetic information and to describe the analytical similarities among these 10 unique compounds (specifically using those methods commonly employed in forensic laboratories). Thus, a methyl silicone stationary phase was used in the initial GC work. We are continuing to try more obvious ways of improving resolution; more detailed studies of the retention properties using both GC and HPLC are underway and will be the subject of a future study.

Acknowledgments

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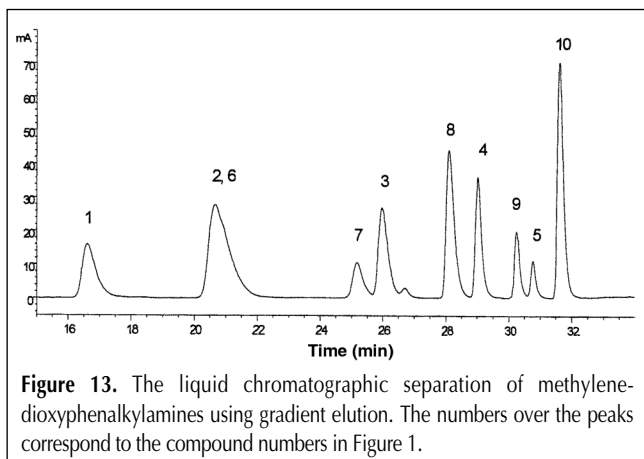**Figure 12.** Influence of the organic solvents on the capacity factor k'.

Figure 13. The liquid chromatographic separation of methylenedioxyphenalkylamines using gradient elution. The numbers over the peaks correspond to the compound numbers in Figure 1.