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Chromatographic Parameter Optimization in LC-MS/MS for the Identification of Indole Isomers (AMT and 5-IT) and Comparative Analysis with Benzodiazepine Compounds.

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Abstract

The identification of isomers, such as AMT (3-(2-aminopropyl) indole) and 5-IT (5-(2-aminopropyl) indole), using LC-MS(/MS) method development presents a significant challenge due to their structural similarities. This experiment aimed to understand the parameters affecting chromatographic separation and explore how modifying these parameters could aid in the differentiation of such isomers that prove difficult for mass spectrometry (MS) to discern. The research involved comprehensive manipulation of chromatographic parameters to improve separation efficiency. By altering the strength of the organic phase, the study demonstrated successful separation, leading to the identification of each drug. However, it was observed that changing the flow rate resulted in a shortened analysis time but potentially compromised the resolution. In contrast, the benzodiazepines method required a different approach. Although complete chromatographic separation was unnecessary, gradient elution proved essential due to the wide range of polarities exhibited by the components. Changing parameters in the benzodiazepine method is to see if the phenomenon is the same for all drugs by changing the strength of the solvents.

This approach allowed for efficient separation within a shorter time period without sacrificing resolution for early peaks or causing excessive broadening of later peaks. Overall, this study enhances the understanding of chromatography and LC-MS(/MS) method development, enabling researchers to successfully differentiate isomers that were previously challenging for mass spectrometry alone. By applying appropriate chromatographic parameters, scientists can enhance the capability of LC-MS(/MS) analysis and contribute to improved analytical practices in various fields.

Keywords: *AMT and 5-IT, Benzodiazepines, LC-MS(/MS), chromatographic parameters.*
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Introduction

LC/MS is an important analytical tool and is also used most often because of its high sensitivity and selectivity. It is used for the separation and detection of molecules. It has a wide variety of applications and is particularly used for the qualification and quantification of analytes of a complex mixture 6.

Liquid chromatography is an essential separation technique used in life sciences and other related branches of chemistry. Liquid chromatography has an advantage over gas chromatography, as it cannot be used for non-volatile and thermally labile compounds. Liquid chromatography is used for the separation of a wide variety of organic compounds. The detectors used for liquid chromatography include refractive index detectors, electrochemical detectors, fluorescence detectors, and ultraviolet-visible detectors. Some of these detectors produce two-dimensional data. That is, the data obtained from these detectors includes signal strength as a function of time. Some detectors, such as fluorescence detectors and diode-array ultraviolet-visible detectors, produce three-dimensional data. That is, these detectors produce signal strength as well as

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spectra for each point in time. Similarly, mass spectrometers also produce three-dimensional data. Besides signal strength, these detectors produce mass spectral data that contains valuable information about the molecular weight, structure, identity, quantity, and purity of the sample. The mass spectral data also adds specificity and confidence to the results of qualitative and quantitative analyses1.

There are two important reasons for using chromatography besides the separation of chemical compounds:

- Most biologically interesting chemicals exist as isomers. Isomers have the same mass and cannot normally be differentiated by a mass detector, as they have the same peak and retention time.
- When a mixture of chemicals enters the process of ionisation, the chemicals may interact and affect the probabilities of getting properly ionized.

It is normally known as the ion suppression effect. It is normally a problem when detecting one minor, or poorly ionized, chemical in the presence of a large amount of something else, maybe a buffer from sample 2.

Usually, the chromatography method is the reverse phase, the first thing that happens to the flow from the HPLC when it enters the mass detector is that the solvent is squirted through a fine needle and evaporated away, to leave metabolites floating free. If the solvent contains non-volatile salts, they will appear, solid and horrible, and clog up the system.

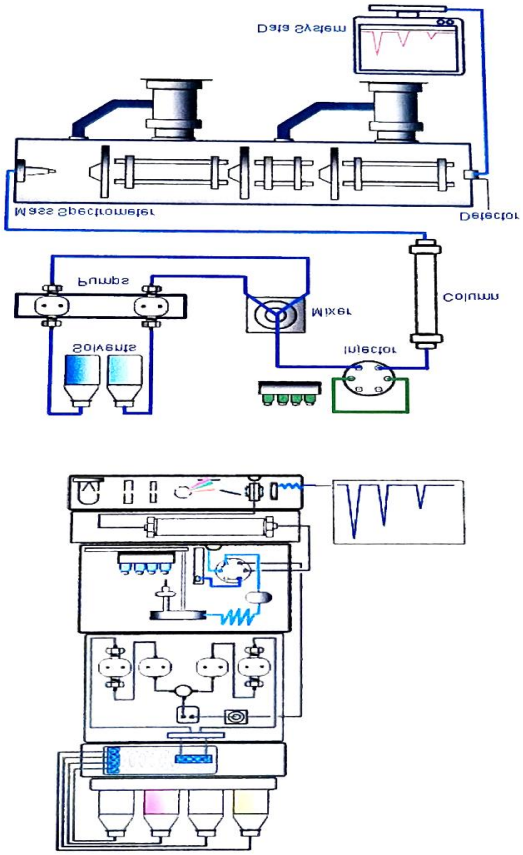

Figure 1: HPLC Diagram

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Therefore, ion exchange chromatography is not really compatible with LC-MS2.

Newer research by local scholars enhances the analytical basis for forensic toxicology and pharmacy research in Duhok. For example, Hussein et al. (2023) pointed out lead toxicity among the workforce, indicating the necessity of consistent toxicological analysis. Also, Hussein and Al-Dosky (2023) pointed to HS-GC-FID as a proven technique in analyzing alcohol for forensic toxicology. From a healthcare perspective, Saeed et al. (2026) stressed the importance of using TESOL communication techniques in pharmacy in Duhok. These works confirm the relevance of the current review concerning the proper methods of analysis and toxicological compound identification.

An LC-MS is basically an HPLC with a Spectra Mass Detector. The HPLC is a system that uses conventional chromatography on a column to separate chemicals. In this case, the metabolite binds to the column by hydrophobic interactions with a hydrophilic solvent, such as water. The metabolite is then washed out by a hydrophobic solvent, such as methanol or acetonitrile. As the metabolites come out of the end of the column, they enter the mass detector. The problem with using just an HPLC is that it will separate things, but it does not really give much information on what a chemical might be. In fact, it is difficult in HPLC to know that a chemical is pure. The addition of a mass spec gives information on what all the chemicals in a peak are, which is a very good way to start identifying them and is a great way to ensure that they are pure. The metabolites must be ionized so that they can be detected by the detector, because it will not work with neutral molecules. The ions must be able to travel through a very good vacuum, so the solvent is removed as a first step. The mass detector then scans all the molecules that it is able to see by their masses, then shows a complete spectrum with all ions that have different masses separated.

Chromatographic Parameters

Some Basic Chromatography Parameters

- Resolution (Rs)
- Retention Factor (k), Capacity Factor (k')
- Selectivity or Separation Factor (a)
- Column Efficiency as Theoretical Plates (N)

Resolution Rs

The most important thing in HPLC is to obtain the 'optimum resolution in the minimum time'. A resolution value of 1.5 or greater between two peaks will ensure that the sample components are well ('baseline') separated, to a degree at which the area or height of each peak may be accurately measured.

Resolution is a measurement of the ability to separate two components.

$$R_S = \frac{(t_{R2} - t_{R1})}{(w_{b1} + w_{b2})/2} = \frac{2(t_{R2} - t_{R1})}{w_{b1} + w_{b2}}$$

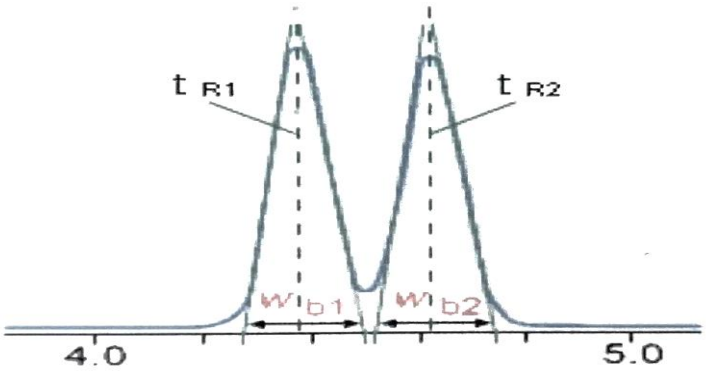

Figure 2: Resolution equation

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Resolution is calculated using the separation of two peaks in terms of their average peak width at the base (t R2 > t R1). In the case of two adjacent peaks, it may be assumed that the peak
width at the base, Wb1 ~ Wb2, and thus, the width of the second peak may be substituted for the average value.

The Fundamental Resolution Equation indicates that resolution is affected by three important parameters: Selectivity (Separation factor), Efficiency, and Retention (Capacity factor)

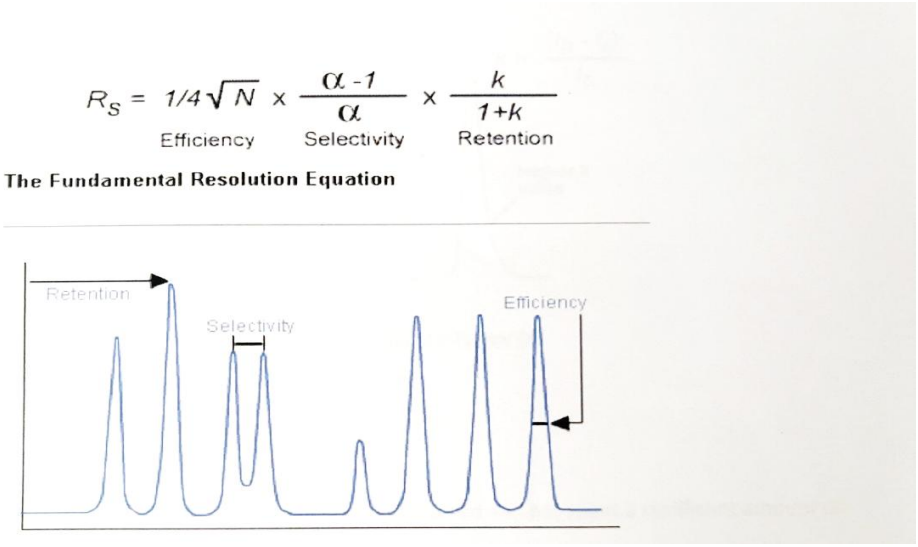

Figure 3: The Fundamental Resolution Equation

N = Column Efficiency - Column length and particle size
a = Selectivity - Mobile phase and stationary phase
k = Retention Factor - Mobile phase strength

Retention (Capacity) Factor (k)

The retention (or capacity) factor is a means of measuring the retention of an analyte on the chromatographic column.

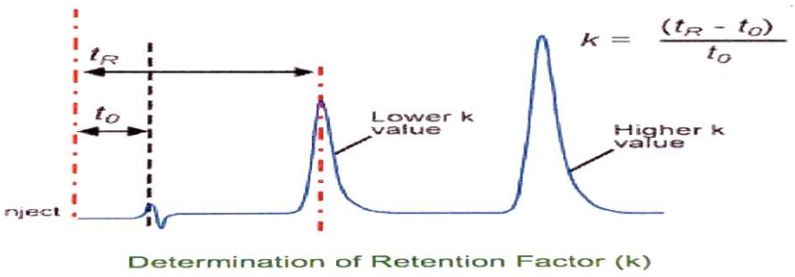

Figure 4: Retention Equation

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A high value of k' suggests that the sample is highly retained and has been in contact with the stationary phase over a long period of time.

The retention factor is given by the ratio of the retention time of the analyte on the column and the retention time of a non-retained compound. The non-retained compound has no retention on the stationary phase and elutes with the solvent front at a time which is also known as the 'hold up time' or 'dead time'. Chromatographers like to have retention factors in the range $1 < k' < 10$.

The most powerful and easiest way of changing the retention factor of a peak is to change the 'Solvent strength' of the mobile phase in the chromatograph. This is usually done in reverse-phase chromatography by changing the proportion of organic modifier in the mobile phase mixture.

As the stationary phase is non-polar (by definition in reverse-phase HPLC), increasing the polarity of the mobile phase will increasingly repel the non-polar parts of the sample molecules towards the stationary and the sample will be retained on the column for a longer period. If the proportion of organic modifier in the mobile phase is increased by 10%, the k value of each component is decreased by a factor of 2-3. This is a powerful separation tool that can be used in the optimization of HPLC separations.

• At low mobile phase organic solvent composition, the retention factor is high, as the analytes are interacting strongly with the stationary phase.

An increase in the organic composition by 10%B results in a 2-3-fold decrease in the retention factor.

• When the retention factors are very high or very low, the quality of separation is poor. Retention factors less than 1, for any of the components, indicate poor separation.

A change in the retention factor (k) values between 1 and 5 has the maximum effect on the separation achieved in the chromatogram.

Selectivity (Separation Factor) (a)

The selectivity (separation factor) (a) is the ability of the chromatographic system to 'chemically' distinguish between the components in the sample. It is usually expressed as a ratio of the retention (capacity) factors (k) of the two peaks being separated and can be visualized as the distance between the apices of the peaks.

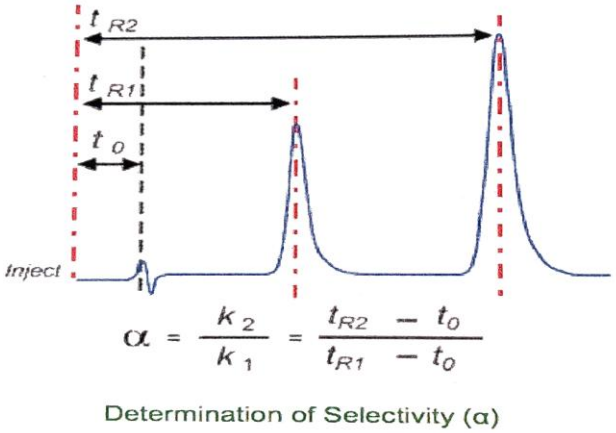

Figure 5: Selectivity Equation

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High values indicate good separating power and good separation; however, a value is NOT directly indicative of the resolution.

The selectivity is always greater than one, as when a is equal to one, the two peaks are co-eluting (i.e., their retention factor values are identical). The greater the selectivity value, the further apart the apices of the two peaks become.

As the selectivity of separation is dependent upon the chemistry of the analyte, mobile and stationary phases, all of these factors may be altered in order to change or optimise the selectivity of an HPLC separation.

There are many factors affecting selectivity (α) in reverse-phase HPLC that can be used to
manipulate the selectivity of HPLC separation

- Organic solvent: Changing to a different solvent (e.g., methanol to acetonitrile in reverse-phase HPLC) will alter the selectivity
- Mobile phase pH: Can alter the degree of ionisation of some analytes - affecting their hydrophobicity
- Solvent strength and additives: Can be adjusted to affect selectivity as well as retention
  (capacity) factor.
- Stationary phase: One of the most popular ways to alter the selectivity of a separation

Temperature: Can have an effect on certain analytes in reverse phase and Chiral HPLC[3].

Effects of Selectivity (α) on Resolution

- Changing selectivity can have a dramatic effect on the chromatographic resolution
- Selectivity is relatively simple to alter, with mobile phase constituents (solvent (type), ion pair reagents, etc.) and pH being the most frequently used methods of adjustment
- If a suitable resolution cannot be achieved by altering the mobile phase constituents, alternative column chemistry should be investigated as a means of altering the selectivity of the separation3.

Efficiency

The efficiency of a chromatographic peak is a measure of the dispersion of the analyte band as it travels through the HPLC system and column.

The plate number (N) is a measure of the peak dispersion on the HPLC column, reflecting the column performance[3].

$$N = 16\left(\frac{t_r}{W_b}\right)^2 = 5.54\left(\frac{t_r}{W_h}\right)^2$$

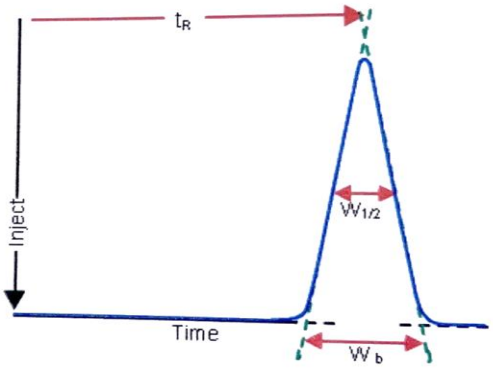

Figure 6 Efficiency Equation

Many factors contribute to the broadening of the peak. The major factor that affects the band broadening (and hence the low efficiency) is the column itself. The packing of the column, the size of the packing, the dimensions of the column, as well as the possibility of the formation of voids

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Original Article

within the column packing, are all factors that affect the efficiency of the column.
• As the column length increases, the peaks get narrower.
• As the efficiency of the peaks increases, the quality of the separated compounds also increases.
• As the length of the column increases, the analysis also takes longer.
• As the length of the column increases by one order of magnitude (from 2.5 to 25cm), the efficiency of the peaks also increases by one order of magnitude.
• 10,000 to 20,000 plates can be achieved with a well-packed column with dimensions 15 x 0.46cm with packing material of 5um.
• Injection volume, dead volume (detector cell, tubing, etc.), and flow rate are also factors that need
to be considered 3.

AMT, 5IT

AMT – 3-(2-Aminopropyl) indole; 5IT – 5-(2-Aminopropyl) indole.
It is very useful to employ LC/MS chromatography techniques and optimize the parameters to identify the compounds as AMT and 5IT. Differentiation between 5-IT and AMT is always a challenging task as the compounds are structurally very similar 5. The isobaric nature of the compounds also does not help as the masses are the same – C11H14N2 – MW – 174.1157 – which cannot be differentiated even with the help of high-resolution accurate mass spectrometry4.

Benzodiazepines

Aims and objectives

This experiment aims to understand the parameters that affect chromatography separation and how, by changing parameters in chromatographic we can separate isomers that are difficult for MS to differentiate. To become more competent with the use of LC-MS(/MS) and to understand the steps involved in LC-MS(/MS) method development.

Experiment

First, check the tuning of the instrument in positive ion mode. The mobile phase, A (1000ml De-H20, 1ml Ammonium acetate2M and 1 ml Formic acid (99-100%)) and B (1000ml Methanol, 1ml Ammonium acetate 2 M and 1 ml Formic acid (99-100%)) were prepared during the previous lab. Second, we prepared solutions for drugs of interest first 0.1 g/ml AMT in 0.1%formic acid, second 0.1 Mg/ml 5IT in 0.1%formic acid, third AMT, and %IT in 0.1% formic acid and finally, Benzodiazepines mixture containing Diazepam, Desmethyldiazepam, Temazepam, Oxazepam, 7-amino flunitrazepam, and their internal standards in 25% methanol:75%de-ionised water supplemented with 2mM ammonium acetate and 0.1% formic acid.
An Agilent 1200 series system interfaced with an Agilent 6400 series triple quadrupole mass spectrometer and ESI ion source was used. For AMT and %IT the sample was run for each drug individually and then the mixture under the initial method for mobile phase 40% A and 60% B, flow rate 0.3ml/min, column temp 40 °C then we altered the parameters by first, changing the organic phase concentration to 80% A and 20 B, and second by changing the flow rate to 0.4 ml/min by fixing the mobile phase at 80%A and 20% B. For the benzodiazepines mixture at the first run under the initial method parameter 40% A + 60% B, flow rate 0.3 ml/min and column temp 40 °C, run for 15 min, then alter the parameters first, we changed the organic phase to 60% A and 40% B, and second, we changed the gradient parameter by changing the B% from 40% to 80% after 4 min and then to 40% again after 9 min with constant flow rate at 0.3 ml/min.

Results

AMT, 5IT

At initial parameters 40%A and 60% B, the AMT and IT run individually and as a mixture to quantify each one, as both have the same precursor and product ions, so they have the same response and
retention time as shown in raw data in the appendix, as they are isomers, but at the initial parameter, there was no separation between both drugs, we had to change the chromatography parameters to help identify the isomer by separating the peaks for both.
For the reverse phase is preferred to start at a high buffer and low organic solvent, first by changing the solvent strength of the chromatographic mobile phase to 80%A and 20% B, decrease the strength of B% organic phase, the retention time table1 was 2.80 for AMT sample and 2.17 for 5IT sample and good separation obtained for both peaks and help to identify the isomer as by comparing the individual peak for each with mixture peaks, the 5IT had peak area 15757 for m/ 158 at 5 collision energy, 9079 for m/ 130 at 21 CE (collision energy) and 1285 for m/ 143 at
26 CE in the same retention time of 2.17, by decreasing the CI, the peak area increased, while AMT had a peak area of 13572 for m/ 158 at collision energy 5, 3216 for m/ 130 at 21 CE, and 4264 for m/ 143 at CI 26, and retention time

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2.83. Second, by changing the flow rate from 0.3 ml/min to 0.4 mi/min while fixing the parameter for organic solvent at 80% A and 20% B, increasing the flow rate, the retention time decreased with good separation peaks, the RT decreased from 2.83 to 2.14, and for 5IT, the retention time decreased from 2.17 to 1.65.

		CI	40%A,60%B	80%A,20%B	0.4ml/min flow rate
AMT	Retention time		No separation	2.83	2.14
	Peak area	5		13572	10949
		21		3216	2553
		26		4264	3552
5IT	Retention time			2.17	1.65
	Peak area	5		15757	12552
		21		9097	7112
		26		4315	1022

Table 1: AMT and 5IT result

Benzodiazepines

The initial parameter running for separating benzodiazepines was at 40% of A and 60% of organic phase B% in the mobile phase. At this parameter, all drugs were identified as they had good separation and good retention time. Changing parameters in the benzodiazepine method is to see if the phenomenon is the same for all drugs by changing the strength of the solvents in the mobile phase to 60% A and 40% B. A big change in retention time for all drugs was observed, as shown in Table 2.

As at low mobile phase organic solvent composition B% is low, the analytes interact strongly with the stationary phase; therefore, the retention time increases, and the peaks become wider.

In both parameters, Diazepam had the greatest retention time. 7-Amino flunitrazepam has the same functional group as diazepam, but it is more polar, so its retention time is less than that of diazepam. By changing the organic phase strength, the retention time increases significantly. Set up a gradient method so 7-amino flunitrazepam comes off at 3.37 min after 4 min in 80% of B. Before that, 40% of B table 3, so 7-amino flunitrazepam did not elute early, the retention time was constant, but after 4- and 9-min, other benzos eluted earlier, and after 9 min the B% decreased to 40% again to avoid any shift in retention time.

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Table 2: Benzothiazines result

		40%,60%	60%,40%	Gradient
Temazepam-D5	RT	4.78	28.92	7.84
Temazepam	RT	4.89	29.79	7.79
Oxazepam-D5	RT	4.33	23.89	7.65
7-aminoflunitrazepam-D7	RT	1.89	3.37	3.37
Diazepam-D5	RT	6.58	43.41	8.21
Oxazepam	RT	4.40	24.46	7.66
Diazepam	RT	6.69	44.84	8.22
7-aminoflunitrazepam	RT	1.91	3.49	3.44
Nordiazepam-D5	RT	5.69	31.77	7.98
Nordiazepam	RT	5.79	32.99	7.99

Table 3: Gradient method parameters

	Time (min)	B%	Flow rate ml/min
1	0	40%	0.3
2	4	40%	0.3
3	4.1	80%	0.3
4	9	80%	0.3
5	9.1	40%	0.3
6	15	40%	0.3

Discussion

AMT and SIT

MRM is an abbreviation for Multiple Reaction Monitoring. In this method, a particular ion, i.e., the precursor ion, is selected from the various ions that are ionized by the ionization probe; the ion is destroyed in the collision cell, i.e., Q2, collision chamber, and a particular ion is detected from the various ions that are destroyed, i.e., the product ions, by Q3. In the LC-MS/MS method, the analysis is done without any

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interference of ions, i.e., the background ions, due to the selectivity of the method, as the analysis is done in two stages. In the LCMSMS method, i.e., the MRM method, the intensity of the analyte peak is low compared to the LCMS, i.e., the SIM method. In the LCMSMS method, the sensitivity of the analysis is higher compared to the LCMS method, as the signal-to-noise ratio is higher due to the low noise level. Therefore, the scan type is important for the analysis of the AMT, 5IT, and benzodiazepines by the ESI ion mode. AMT, 5IT Structural Diagram

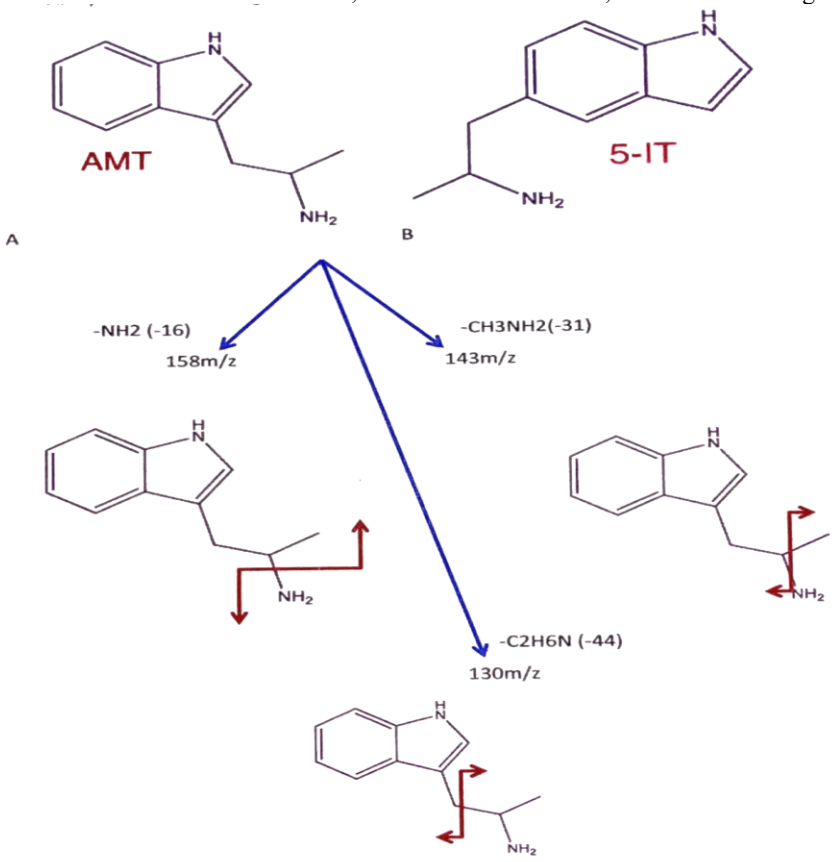

Figure 7 AMT,5IT Fragments, A= AMT, B=5-IT

AMT and 5-IT are isomers, i.e., they have the same structure and MW, hence when it is fragmented, it produces the same fragments as the original compound, and the product ion is similar, hence by the chromatogram, we can separate these isomers, hence we can identify the individual drugs, as AMT is a sedative drug, hence we need to identify it. Even by the chromatography technique, we still need to adjust the parameters in order to obtain good separation. By adjusting the strength of the mobile phase and the flow rate, we obtain good separation with a short analytical time.

Benzodiazepine

Deuterated internal standards have the same extraction recovery, the same ionization response in ESI mass spectrometry, and the same chromatographic retention time. An important feature of a suitable deuterated internal standard is that it co-elutes with the compound of interest. In addition, it should have a sufficient mass shift from the analyte that the signal is outside the natural mass distribution of the analyte. The deuterated internal standard is really useful when we are using the deuterated internal standard in the analysis of diazepam, nordiazepam, temazepam, and

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oxazepam, as the internal standard of diazepam, nordiazepam, temazepam, and oxazepam is similar to the analyte, hence they were eluted at the same retention time, hence chemically similar, hence they produced the same charge, hence the instrument is easily able to distinguish the two signals, as they are similar in most aspects but different in some aspects, hence the signal is easily distinguishable.

Changing the organic solvent content of the mobile phase had a great effect on the separation of diazepam, nordiazepam, temazepam, and oxazepam, as the peak became broader and less sharp, and decreased the peak area as well. However, the benzodiazepine drugs are separated and identified as the complete chromatographic separation is not necessary, as there are no isomers between the drugs.

Gradient elution: Two or more solvent systems are used that vary significantly in polarity. Once the elution is started, the proportion of the two or more solvents is varied in a programmed manner that is either continuous or stepped. The efficiency of the separation is greatly improved by using the gradient elution technique. The technique of choice is when the sample contains components with a wide range of polarities. When using the reverse phase gradient elution, the solvent is initially polar and becomes less polar with the passage of time. The gradient elution technique gives the best separated peaks without taking too long a time. It can also separate the components of the sample containing compounds with a wide range of polarities within a short time period without the resolution of the peaks being lost in the initial part of the chromatogram or the peaks becoming excessively broad in the later part of the chromatogram. However, the gradient elution technique also has the disadvantage of requiring more expensive and complicated equipment, as well as difficulty in maintaining the constant flow rate, as the composition of the mobile phase is constantly changing. The gradient elution technique, especially at high speeds, also points out the limitations of the lower quality of the experimental apparatus, as the reproducibility of the results obtained is less due to the tendency of the apparatus to vary anyway. In addition, if the flow rate changes or the composition of the mobile phase varies, the results will not be reproducible.

Conclusion

In both cases of AMT and SIT, mass spectrometry cannot distinguish between them because they are isomers. So, by changing the chromatography parameters and using complete separation, we can distinguish each drug. By changing the strength of the organic phase, we got good separation, but by changing the flow rate, we got a shortened period of time as well. But in the benzodiazepines method, there is no need to use complete chromatographic separation; gradient elution is required because the components have a wide range of polarities that can be separated using gradient elution in a shorter time period without resolution being compromised in the earlier peaks or excessive broadening of later peaks.

Acknowledgements

The authors would like to thank the College of Pharmacy, University of Duhok, and Charsteen Institute for their support in academic and technical aspects in the preparation of this research.

List of Abbreviations

AMT 3-(2-Aminopropyl)indole
5-IT 5-(2-Aminopropyl)indole
LC-MS Liquid Chromatography–Mass Spectrometry
LC-MS/MS Liquid Chromatography–Tandem Mass Spectrometry
HPLC High-Performance Liquid Chromatography
MS Mass Spectrometry
MRM Multiple Reaction Monitoring
ESI Electrospray Ionization
RT Retention Time
CE Collision Energy
CI Collision-Induced Fragmentation
MW Molecular Weight
Rs Resolution
k / k′ Retention or Capacity Factor
α Selectivity or Separation Factor
N Column Efficiency / Theoretical Plates

Authors' Contributions

Jwan Hasan Hussein contributed to the conceptualization of the research, designing the research, laboratory methodology development, interpreting the results of the chromatography and LC-MS/MS, writing the manuscript, and revising the manuscript.

Nirozh Azad Chalabi participated in experimenting, preparing the analytical solution, collecting the data, interpreting the results of LC-MS/MS, supporting with literature, and reviewing the manuscript.

Both authors read and approved the final manuscript and agreed to be responsible for the accuracy and integrity of the work.

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Data Availability Statement

The datasets generated and/or analyzed during the current study are presented in this article. Additional raw data may be requested from the corresponding author.

Conflict of Interest

The authors declared no conflict of interest regarding the publication of this paper.

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