# Determination of Tramadol, Metamizole, Ropivacaine, and Bupivacaine in Analgesic Mixture Samples by HPLC with DAD Detection

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## Abstract

**A high-performance liquid chromatography–diode array detection method was developed and validated to simultaneously determine tramadol (TMD), metamizole (MTZ), ropivacaine (RPV), and bupivacaine (BPV) in the presence of 4-methylaminoantypirine (4-MAA), the metabolite of MTZ, in analgesic mixtures samples used in Patient Controlled Analgesia (PCA). Chromatographic separation is achieved with a C-18 column using a mixture of ACN–methanol–water adjusted to pH 3.0 with $NaH_2PO_4$ 0.05M (10:25:65 v/v) in an isocratic mobile phase at a flow rate of 0.8 mL/min. 0.5 mg/mL of $Na_2SO_3$ in the water of the mobile phase was necessary to prevent the fast MTZ hydrolysis process to 4-MAA. Ultraviolet-diode array detection was used and chromatograms were registered at the wavelength of 230 nm. The method was linear in the range of 2.2–80.0 mg/L for TMD, 4.1–140.0 mg/L for MTZ, 2.3–40.0 mg/L for RPV, and 2.9–40.0 mg/L for BPV. Validation of the method was made in terms of accuracy, intra- and interday precision, as well as quantification and detection limits. The hydrolysis of MTZ to 4-MAA was studied and verified by mass spectrometry. The developed method was used successfully to evaluate the chemical stability of binary analgesic TMD mixtures with MTZ, RPV, or BPV. The mixtures were tested at standard concentrations used in PCA and in different storage conditions. When mixtures contained MTZ, a chromatographic peak from the metabolite 4-MAA was always detected in the chromatograms.**

## Introduction

Combinations of different drug solutions are often used in clinical practice to relieve post-operative pain using the Patient Controlled Analgesia (PCA) (1,2) technique, although little or no information is available about the chemical stability and compatibility of these analgesic mixtures. Currently, there are no commercially available analgesic mixtures, and they must be prepared in the hospital pharmacy on an "as-needed" basis. The mixture composition depends on the sort of surgery performed and the intensity of the pain. Several of these drug mixtures used for this purpose are composed of tramadol (TMD) with other drugs, such as metamizole (MTZ), ropivacaine (RPV), and bupivacaine (BPV) (Figure 1).

TMD (trans(±)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol) is a synthetic, centrally-acting analgesic agent. The drug acts as an opiate agonist by selective activity at the μ-opioid receptors. In addition to opiate agonist activity, TMD inhibits the reuptake of neropinephrine and serotonin, which appears to contribute to the drug's analgesic effect (3). MTZ (sodium [N-(1,5-dimethyl-3-oxo-2-phenylpyrazolin-4-yl)-N-methylamino]) is a pyrazolone non-steroidal anti-inflammatory drug (NSAID). It is a potent analgesic and antipyretic drug, which has been used for more than 70 years. It is indicated for severe pain and for gall bladder and kidney colic and is commonly used in several countries in Europe, Asia, and South America, being the first line analgesic in numerous developing countries (4). In contrast to this, MTZ has been banned in the US, UK, and Sweden because of its adverse drug reactions, notably agranulocytosis and anaphylactic shock reactions (5). In Germany, its use is restricted to special kinds of diseases. Nevertheless, the problems associated with the administration of MTZ are not completely clear today (6). Even the entire mecha-

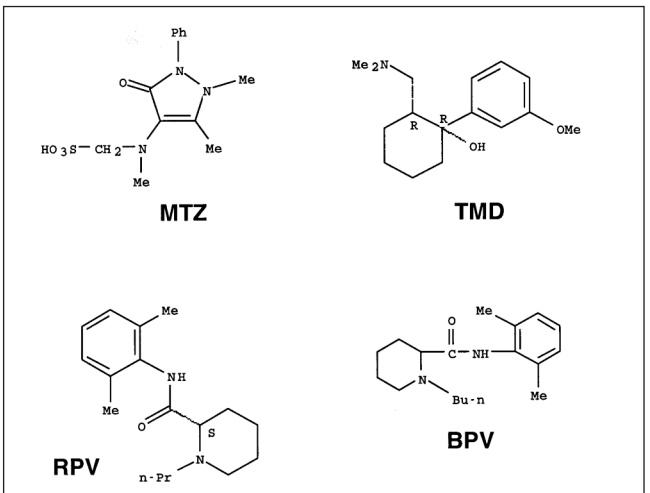


**Figure 1.** Structures of the molecules studied.

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nism of the action of MTZ is still unknown (7). But it is well known that MTZ is rapidly and non-enzymatically hydrolyzed to its main metabolite, 4-methylaminoantypirine (4-MAA) (8–10). The analgesic effect of MTZ has been correlated to the concentration of 4-MAA (11), but the contribution of other metabolites to the effects of MTZ treatment is poorly understood. RPV (N-(2,6-dimethylphenyl)-1-propyl-,(2S)-2-piperidinecarboxamide) and BPV (1-butyl-N-(2,6-dimethylphenyl)-2-piperidinecarboxamide) are both long-acting local anaesthetic drugs with similar pharmacodynamic properties, mainly used in surgery and for post-operative pain relief. RPV is synthesised as the pure S-enantiomer and differs from BPV chemically in having a different alkyl group (1-propyl alkyl group substituted for the 1-butyl group) and also in being a pure enantiomer, one of the reasons for its lower toxicity.

Because these drugs are widely used, many high-performance liquid chromatographic (HPLC) methods have been proposed to analyze them, both when single or in the presence of metabolites and other drugs. For TMD, recent methods propose the simultaneous determination of the drug and its metabolites in human plasma (12,13) and the determination of the drug and its impurities in oral drops (14). TMD was also analyzed in human breast milk (15). Additionally, an HPLC method to study the stability of benzalkonium chloride in 0.5% TMD ophthalmic solution was reported (16). A few methods claim to determine MTZ by HPLC (17,18), but without taking into account the spontaneous hydrolysis process of MTZ to 4-MAA. When MTZ was determined in Pentalgin tablets by HPLC, the use of sodium sulphite was proposed to prevent the MTZ hydrolysis process (19). Recently, a new kinetic-chromatographic determination of the hydrolysis product of MTZ (4-MAA) in pharmaceutical commercial products has been reported (20). A permanent matter of interest is the determination of local anaesthetic drugs like RPV and BPV. HPLC is the most often used technique for this purpose (21–26). Several stability and compatibility studies of this mixture with other drugs were also published (27–30). The simultaneous determination of RPV and BPV by HPLC was developed and applied to human plasma samples (31,32). Nevertheless, to the best of our knowledge, there is no method to simultaneously determine the four drugs studied in the present work.

In this study, a rapid, reproducible, and sensitive HPLC method was developed and validated to determine TMD, MTZ, RPV, and BPV in the presence of the main non-enzymatic metabolite of MTZ, 4-MAA. These drugs are widely used in clinical practice. The proposed method was successfully applied to analyze binary mixture samples of the drugs at the usual concentrations used in PCA in order to test their stability in different conservation conditions.

## Experimental

### Chemicals and reagents

The standard sample of TMD chlorhydrate was supplied by Laboratorios Normon, S.A. (Madrid, Spain); magnesium MTZ was supplied by Boehringer Ingelheim S.A. (Barcelona, Spain); BPV was supplied by Laboratorios Inibsa S.A. (Barcelona, Spain); and RPV by AstraZeneca International (Sodertalje, Sweden). The pharmaceutical formulations used to prepare the mixtures were Nolotil ampoule (2 g/5 mL MTZ-Mg), Boehringer Ingelheim; Adolonta (100 mg/mL TMD), Laboratorios Normon, S.A.; Svedocain 0.50% (5 g/10 mL BPV), Inibsa; and Naropin Polyamp injectable solution (10 mg/mL RPV); AstraZeneca. The solution of 0.9% NaCl used to prepare the sample mixtures was from Fisiológico Braun (B. Braun Medical S.A., Spain).

All reagents were of analytical reagent grade unless stated otherwise. Reverse-osmosis type quality water (purified with a Milli-RO plus Milli-Q station from Millipore, Milford, MA) and HPLC quality were used throughout. Acetonitrile, methanol, monosodium dihydrogen phosphate, and sodium sulphite were supplied by Panreac (Barcelona, Spain).

### Preparation of standards

Individual stock standard solutions of TMD, RPV, and BPV of 1.0 g/L were prepared by dissolving each pure compound in water. These solutions were stored in dark bottles at –4°C and warmed up to room temperature before use. The stability of these solutions was tested to be at least four months. The working standard solutions were prepared daily in amber-colored vials by diluting the standard solutions in 0.7 mg/mL $Na_2SO_3$ solution. An individual stock standard solution of MTZ of 1.0 g/L was prepared in 0.7 mg/mL $Na_2SO_3$ solution. This solution was tested to be stable for a week. The working standard solutions were prepared daily in amber-colored vials by diluting the standard solutions in 0.7 mg/mL $Na_2SO_3$ solution. Calibration graphs adjusted to the problem studied were established between 40.0 and 140.0 mg/mL for MTZ, between 2.2 and 80.0 mg/mL for TMD, between 2.3 and 40.0 mg/mL for RPV, and between 2.9 and 40.0 mg/mL for RPV.

### Preparation and conservation of the samples

Sterility conditions in a laminar flow extractor (100 Telstar BH 10, Barcelona, Spain) were used to prepare three analgesic mixture solutions containing 5.0 mg/mL TMD and 100.0 mg/mL MTZ, 4.0 mg/mL TMD and 2.0 mg/mL RPV, and 4.0 mg/mL TMD and 1.2 mg/mL BPV, all of them in 0.9% NaCl solution. From each solution and using a peristaltic pump (Baxa Repeater Pump, Madrid, Spain), 15 aliquot solutions of 20 mL were placed in the polyolefin bags. One solution from each mixture was analyzed immediately after its preparation, and the concentration determined was the initial concentration for further studies. Batches of four bags for every mixture were placed in the different conservation conditions studied: (*i*) stored at room temperature in daylight; (*ii*) stored at room temperature in darkness; and (*iii*) stored at 4°C in darkness. One bag from each batch was analyzed 1, 3, 7, and 15 days after its preparation.

### Chromatographic conditions

Chromatography was performed using an 1100 Agilent HPLC coupled with an 1100 Agilent Photodiode Array Detector (Madrid, Spain). The degasser and the autosampler systems were also 1100 Agilent. The equipment was connected to a Pentium 200 fitted with an HPLC ChemStation workstation. TMD, MTZ, RPV, and BPV were separated by HPLC on a 250 mm × 4.6 mm i.d., 5 µm particle size ODS analytical column (Agilent Technologies), using a mobile phase containing acetoni-

trile–methanol–water adjusted to pH 3.0 with $NaH_2PO_4$ 0.05M (10:25:65 v/v), at a flow rate of 0.8 mL/min. To prevent MET hydrolysis to 4-MAA, 0.5 mg/mL of $Na_2SO_3$ were added to the water of the mobile phase. The temperature of the column was maintained at 25°C. 15 µL was used as the injected volume, and the chromatograms were registered at 230 nm using 360 nm as the reference wavelength.

The pH was measured in a digital pH-meter from Crison model Basic 20 (Barcelona, Spain). Samples were filtered with a 45 µm filter from Waters (Madrid, Spain) before being injected into the chromatograph system. Software programs used for the treatment of the chromatographic data were the Statgraphics Plus 6.0 software package (Statistical Graphics System, USA, 1992) and Excel Software package (Microsoft Office XP).

### Mass spectrum conditions

A Z-Spray Mass Detector Micromass Platform ZMD-4000 (Waters Corporation, Milford, MA), with an electrospray interface was used to record mass spectra. The equipment was connected to a Pentium IV 3,00 *GHz* fitted with a MassLynx workstation. The automatic syringe bomb used to inject the samples directly into the mass detector was from Harvard Apparatus (Holliston).

The mass spectrometer was operated in the electrospray ionisation mode (ESI) with positive ion detection. High-purity nitrogen was used as the drying gas (gas flow 300 L/h) and as the ESI nebulising gas, with the pressure set at 200.0 psi. The source block and the desolvation temperature were set at 100°C and 400°C, respectively. The capillary voltage was set at 2.50 kV, and the cone voltage was set at 10 V. The tube lens voltage and the ion energy were set at 0.5 V and 0.5 V, respectively.

### Validation of the method

Validation of the method was made in terms of linearity, accuracy, intra- and inter-day precision, and quantification and detection limits for each analyte. The linearity of the method was checked using sets of up to five concentration levels. Linearity was accepted if the P-value of the corresponding lack-of-fit test was greater than 5% according to the guidelines of the Analytical Methods Committee (33). Accuracy and intraday precision were estimated by means of the recovery value and relative standard deviation (RSD, %) calculated from 10 replicate mixture samples prepared from the standards, at concentration levels placed in the middle of the calibration functions. Interday precision (5 days) was also estimated as the RSD calculated from five replicate mixture samples prepared in the same way. Quantification and detection limits were estimated from ten and three times the signal/noise ratio, respectively.

### Chemical stability study of the analgesic mixtures

In the chemical stability study, the concentrations of the drugs were expressed as the percentage of the remaining drug concentration at different times for every drug in the mixtures under the different conservation conditions studied. In all cases except for MTZ, the measured concentration was the average of four replicates. In this study, stability was assumed if the loss was less than 10% of the initial concentration. In the case of MTZ, because the mixture samples were prepared and stored without adding $Na_2SO_3$—as usual in clinical practice—the MTZ hydrolysis process gave no reproducible analysis. The first of four analyses was used to build the MTZ graphs.

## Results and Discussion

### Development and validation of the HPLC method

From the consulted literature, acetonitrile, methanol, and an acidic aqueous medium were selected to start the optimization of the mobile phase composition. Phosphoric acid, $NaH_2PO_4$, and $Na_2HPO_4$ were selected to adjust the pH of the mobile phase at acidic values between 3 and 6. Increasing values of the pH gave larger retention times for TMD, RPV, and BPV, with MTZ being the drug less affected by the pH change. pH 3 and 4 gave similar retention times, although the peak shape was narrower as the pH decreased for all the drugs. Therefore, pH 3.0 was selected. This pH value was adjusted using $NaH_2PO_4$ at a concentration of 0.05 M. Acetonitrile and methanol were tested at different percentages in the mobile phase. As expected, all the drugs eluted at shorter retention times when the percentage of the organic solvents increased. Obviously, this effect was slightly higher for acetonitrile, as it is less polar. Finally, setting the acetonitrile percentage at 10%, and methanol percentage at 25% in the mobile phase, it was possible to achieve a good chromatographic separation for the four drugs (Figure 2).

As is well-documented in the references, MTZ is a pro-drug that hydrolyses quickly to 4-MAA (7,8,14,19). This transformation occurs both in water and methanol, being faster in water and at acidic pH values (14). 4-MAA presents the same pharmacologic effects as MTZ. In fact, the 4-MAA is the form in which MTZ acts because it is not enzimatically completely hydrolyzed 5 min after oral ingestion (34). It was then necessary to add $Na_2SO_3$ to all the solutions containing MTZ (stock standard solutions and working standard solutions) (19) and to the mobile phase, to prevent the hydrolysis of MTZ, because tests showed that if no sodium sulphite was added when MTZ was present, the MTZ hydrolysis to 4-MAA was produced even during the chromatographic process. A small peak assigned to 4-MAA was placed in the chromatogram between peaks of MTZ and TMD at around 5 min (Figure 2) when $Na_2SO_3$ was added to the standard samples but not to the mobile phase. This fact is seen in the hydrolysis of MTZ during the chromatographic process with this transformation helped by the acidic pH value (pH 3) of the mobile phase. For the highest concentration used of MTZ (1.0 g/L) in the standard samples, tests showed that 0.7 mg/mL $Na_2SO_3$ was enough to prevent its hydrolysis. Thus, this $Na_2SO_3$ concentration was always used in the solutions containing MTZ. The concentration of $Na_2SO_3$ in the mobile phase was tested between 0.5 mg/mL and 1.5 mg/mL. Concentrations higher than 0.7 mg/mL gave a $Na_2SO_3$ chromatographic peak at around 3.3 min, a retention time that overlapped with the MTZ peak (around 4.4 min). Therefore, 0.5 mg/mL was selected as $Na_2SO_3$ optimum concentration to add to the mobile phase. Since 0.7 mg/mL were selected to guarantee no hydrolysis process in the standard MTZ solutions, a small peak from $Na_2SO_3$ was always detected in the chromatograms when these solutions were analyzed.

In the selected chromatographic conditions described earlier, the retention times were: 3.3 min for $Na_2SO_3$, 4.1 min for MTZ, 5.1 for 4-MAA, 6.7 for TMD, 9.0 for RPV, and 12.4 for BPV, with a total run time of 15 min. In this way, it was possible to achieve good chromatographic separation not only for the four drugs studied, but also for the 4-MAA and for the $Na_2SO_3$ used as MTZ preservative.

A UV–DAD was used because it offers more advantages than the conventional UV detector. It makes it possible to check the peak purity by scanning the full spectrum that emerges from the chromatographic column and to record simultaneously at maximum wavelength. For the four drugs studied, chromatograms were registered at the same wavelength since all of them exhibit a UV maximum at the similar wavelength of 230 nm.

The concentrations studied for the calibration functions were established, taking into account the concentrations of the drugs in the three mixture samples used in PCA. These concentrations were: 5.0 mg/mL TMD/100.0 mg/mL MTZ; 4.0 mg/mL TMD/2.0 mg/mL RPV; and 4.0 mg/mL TMD/1.2 mg/mL BPV. The dilutions applied to the mixture samples were 1/1000, 1/100, and 1/100, respectively. In accordance with this dilution, the concentrations to be analyzed in the samples were 40.0 mg/L (in mixtures with RPV and BPV) and 5.0 mg/L (in mixtures with MTZ) for TMD; 100.0 mg/L for MTZ; 20.0 mg/L for RPV; and 12 mg/L for BPV. Then, the upper limits of the calibration functions were adjusted to the drug concentration in the problem. Five concentration levels and four replicates for each one were used to characterise the calibration functions. The linearity of these functions was tested to be between 4.1 and 140.0 mg/L for MTZ, between 2.2 and 80.0 for TMD mg/L, between 2.3 and 40.0 mg/L for BPV, and between 2.9 and 40 mg/L for BPV. The lack-of-fit test was applied to check the linearity. The results for the intercepts (a), slopes (b), and correlation coefficients ($R^2$) as well as the probability levels of the lack-of-fit test (P %) are summarized in Table I. The results were indicative of good linearity within the range under study with a correlation coefficient higher than 99.00% and probability levels up to 97%, demonstrating the good linearity of the response of the detector in the selected conditions for each drug. The intercepts were not significant because the corresponding P-values were greater that 0.05%. The detection and quantification limits were calculated from the signal/noise ratio as usual in chromatographic methods, with the former being between 2.2 and 4.1 mg/L and the latter between 0.2 and 1.2 mg/L. The results are shown in Table I.

As shown in Table II, the results obtained for the accuracy and the intra- and inter-day precision of the method, expressed as recovery and RSD % values, were satisfactory. As shown in this table, the intra- and inter-day RSD % were below 2.5% for all the drugs, with the recoveries obtained also being close to 100%.

**Table I. Analytical Parameters**

| | MTZ | TMD | RPV | BPV |
|---|---|---|---|---|
| Intercept (a) | –34.1 | –41.1 | –65.7 | –40.2 |
| Slope (b) | 37.8 | 43.4 | 36.6 | 30.2 |
| s (a)* | 3.2 | 0.74 | 0.9 | 1.0 |
| a, P-value (%)† | 65.52 | 72.16 | 68.49 | 84.25 |
| s (b)‡ | 0.03 | 0.02 | 0.04 | 0.04 |
| $R^2$§ (%) | 99.998 | 99.999 | 99.998 | 99.798 |
| P-value (%)** | 79.44 | 87.14 | 96.53 | 75.28 |
| Linear dynamic range (mg/L) | 4.1–140.0 | 2.2–80.0 | 2.3–40.0 | 2.9–40.0 |
| Quantification limit†† (mg/L) | 4.1 | 2.2 | 2.3 | 2.9 |
| Detection limit†† (mg/L) | 1.2 | 0.2 | 0.7 | 0.8 |

* Standard deviation of the intercept.
† Probability of intercept significant.
‡ Standard deviation of the slope.
§ Correlation coefficient.
** Probability of lack-of-fit test.
†† Calculated from noise-to-signal ratio from blank samples.

**Table II. Precision and Accuracy**

| Compound | Concentration tested (mg/L) | Recovery* (%) | Precision RSD (%)† | |
|---|---|---|---|---|
| | | | Intra-day | Inter-day (5 days) |
| MTZ | 100.0 | 99.3 | 1.7 | 2.4 |
| TMD | 40.0 | 99.4 | 0.7 | 1.5 |
| RPV | 20.0 | 99.6 | 1.2 | 1.8 |
| BPV | 20.0 | 100.9 | 1.5 | 2.1 |

* Recovery value obtained from ten samples prepared from the standard.
† Relative standard deviation from ten standard samples.

### MTZ hydrolysis to 4-MAA

In order to confirm the hydrolysis of MTZ to 4-MAA, a solution of 1.0 g/L of MTZ was prepared in water and stored in a glass bottle at room temperature for a month. A mass spectrum of this solution was taken a month after its preparation. In this mass spectrum (Figure 3), the presence of two peaks can be noticed, the first at 218 *m/z* and the second at 240 *m/z*. These peaks were assigned to the molecular peak of 4-MAA, $[4\text{-MAA} + H]^+$ and the adduct of the 4-MAA with Na, $[4\text{-MAA} + Na]^+$, respectively. No signal of MTZ (MW: 333 g/mol) was found in the mass spectrum (Figure 3), so it was demonstrated that the hydrolysis process of MTZ to 4-MAA was complete in a month. From the visual analysis of the *m/z* spectrum, it was also tested that no other metabolites from MTZ different from 4-MAA were produced in the spontaneous non-enzymatic hydrolysis process.

### Chemical stability study of analgesic mixtures

The HPLC–DAD method described earlier was applied to study the chemical stability of the analgesic mixtures prepared in isotonic conditions and in different storage conditions. Figure 4 presents the results of this study.

Comparing the evolution of TMD concentration in the three mixtures studied (Figures 4A, 4C, and 4D), it can be observed that its behavior was similar in all of them, regardless of the conservation method during the time tested, 15 days. TMD degradation was not observed because no unassigned peak was found in the

corresponding chromatograms of TMD mixtures. Recovery values ranged from 96.0% to 104.3%, and the RSD values were less than 3.6%. It can be noted that instead of the high dilution needed to analyze the mixture samples (1/100 for mixtures with RPV and BPV, and 1/1000 for mixtures with MTZ), the RSD values were successful.

RPV (Figure 4D) and BPV (Figure 4F) showed similar behavior in their respective mixtures with TMD in the different conservation conditions tested. Again, unassigned peaks in the chromatograms from the mixture samples were not detected where they were present. For RPV, recovery values ranged from 96.5% to 106.2%, with RSD less than 5.0%; and for BPV recovery values were between 92.6% and 103.8%, with RSD less than 5.0%.

On the contrary, MTZ showed an evident decrease of its concentration in its mixture with TMD (Figure 4B). This decrease

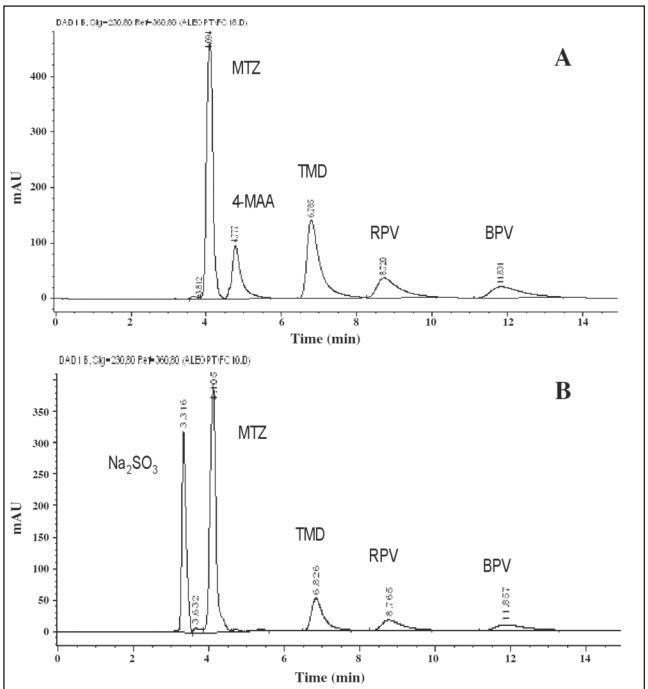


**Figure 2.** Chromatograms of standard drug mixture samples, without $Na_2SO_3$ in the mobile phase; 140 mg/L MTZ, 80 mg/L TMD, 40 mg/L ROP, and 40 mg/L BUP (A); and adding $Na_2SO_3$ (0.5 mg/mL) in the mobile phase; 100 mg/L MTZ, 30 mg/L TMD, 20 mg/L ROP, and 20 mg/L BUP (B).

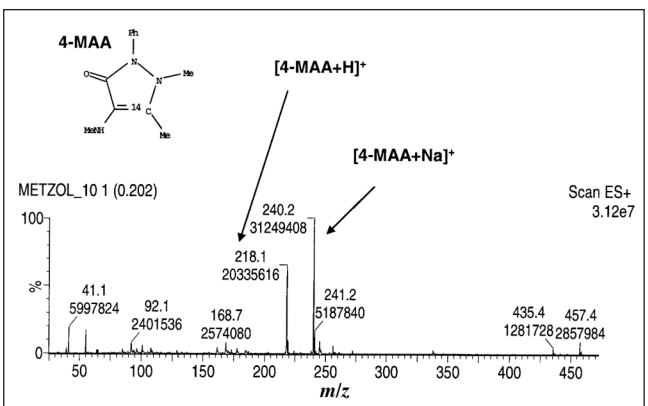


**Figure 3.** Mass spectrum of a water solution of MTZ a month after its preparation. The 4-MAA spectrum can be identified by the peak assignments.

was independent of the presence of TMD, and it was the result of the well-known and already cited hydrolysis process of MTZ in the 4-MAA (pharmacologically active compound) in aqueous medium. The TMD/MTZ mixture samples were prepared as they are used in PCA, without adding sodium sulphite, and the hydrolysis was then produced. It was also a process independent of the conservation conditions; in samples kept protected from daylight at 4°C, the hydrolysis of MTZ was observed. The hydrolysis was a fast process that began immediately after the preparation of the MTZ aqueous solution. Replicates from samples analyzed immediately after their preparation were not reproducible since the MTZ concentration was decreasing between chromatograms, thus the RSD values were not calculated in the analysis of the samples and only the first replicate was used to draw the MTZ graph (Figure 4A and 4B). Figure 5 shows the areas under the chromatographic peaks of MTZ and 4-MAA corresponding to four analyses carried out in the same sample, right after the preparation of the TMD/MTZ mixture; it is a good graphic presentation of the hydrolysis process of MTZ. The prolonged stability of dipyrone's commercial water formulations

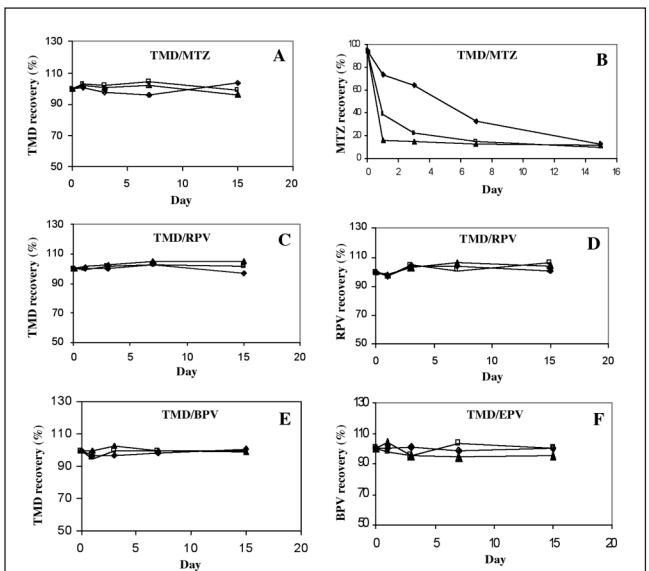


**Figure 4.** Chemical stability results. Code lines: ♦, samples stored at room temperature in daylight; □, stored at room temperate in darkness; ▲, stored at 4°C in darkness.

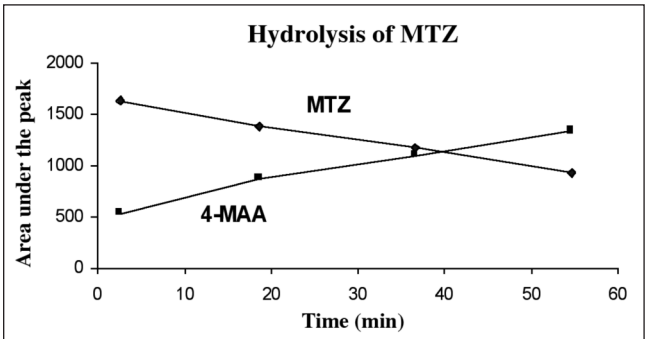


**Figure 5.** Graphic presentation of the hydrolysis of MTZ: data are presented as area under the chromatographic peak for MTZ and 4-MAA in the same sample, analysed right after the preparation of the TMD/MTZ mixture (day 0 in the stability study).

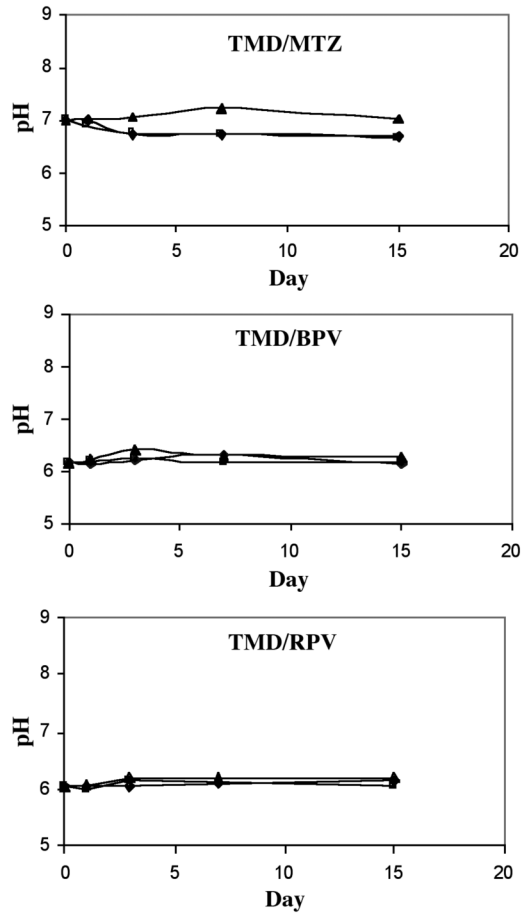


**Figure 6.** pH study results. Code lines: ♦, samples stored at room temperature at daylight; □ , stored at room temperate in darkness; ▲, stored at 4°C in darkness.

(5 years at a concentration of 2 mg/5 mL) can be explained by its high concentration (10) because the concentration is the major factor in the hydrolysis of MTZ and an increasing concentration of MTZ decreases the rate of hydrolysis.

The concentration tested of MTZ (100.0 mg/L) in the mixture samples was considered as the initial concentration to calculate the percentage of the remaining concentration. When MTZ mixtures were stored at room temperature and without protection from daylight, the remaining MTZ concentration was less than 65%, three days after its preparation. When stored at room temperature without protection from daylight, this percentage fell to 40%, and when stored at 4°C with protection from daylight, this percentage decreased drastically below 15%. In all the conservation conditions tested, the percentage of the remaining concentration after 15 days of the sample preparation was around 10%. Curiously, samples stored at 4°C and protected from daylight were the most affected by the hydrolysis process, while those stored at room temperature without protection from daylight were less affected (Figure 4B).

The pH of the mixture samples was also tested during the study (Figure 6). Mixtures of TMD with RPV and BPV showed a similar behavior, with the pH value close to pH 6 in all the stored conditions tested. For the mixture of TMD with MTZ, the pH was close to 7. The hydrolysis process of MTZ to 4-MAA was not affected by the pH value (20). Regarding the pH value of the mixture samples during the 15 days studied, it can be concluded that this value was acceptable in all situations studied for the drug mixture to avoid clinical problems such as phlebitis or intravenous tract irritability.

### Physical compatibility study of the mixture samples

Additionally, the physical compatibility of the analgesic mixture samples was tested. This study was carried out by visual observation of possible physical alterations, such as color changes and the appearance of precipitated or muddy samples. The visual testing was performed by direct sample observation on a black and white background and by comparing the drug mixture samples with 0.9% NaCl medium. The physical changes were tested on the first day and on days 1, 3, 7, and 15.

For the TMD mixtures with RPV and BPV, no instability signal was observed for any situation studied, for example no precipitated or color change was detected. At least after 15 days from its preparation, and under the different storage conditions tested, the physical compatibility of the drug mixture was corroborated. For the TMD mixtures with MTZ, the samples took on a yellowish color a few min after their preparation. The intensity of the color increased with time, regardless of the storage conditions. This behavior is characteristic of the aqueous solutions of pyrazolones, such as MTZ, and the color is due to the hydrolysis products (35). No precipitated or muddy samples were observed in any case.

## Conclusion

A new and validated analytical HPLC–DAD method for the simultaneous determination of TMD, MTZ, RPV, and BPV, in the presence of the main active no-enzymatic metabolite of MTZ, 4-MAA, has been successfully developed. The method preserved the spontaneous hydrolysis process of MTZ to 4-MAA during the analysis, and it was successfully applied to determine the drugs in their mixture in isotonic medium such as NaCl 0.9%. Next, the method was used to study the stability of the binary mixture of the drugs at the usual concentration levels used in PCA. Regarding this stability study, the results showed that the drug mixtures TMD/RPV and TMD/BPV both prepared in 0.9% NaCl and stored in Viaflo bags for 15 days, were chemically stable and physically compatible in the different conservation conditions studied. Thus, it can be concluded that these drug mixtures are pharmaceutically stable in that medium and suitable for use in PCA for at least 15 days after their preparation. The mixture of TMD with MTZ in 0.9 % NaCl solution showed more complex characteristics due to the presence of MTZ, which hydrolyses to 4-MAA. Nevertheless, this compound exhibits the same pharmacological characteristics as MTZ. Therefore, it can be concluded that rather than this mixture not being chemically stable, it could be considered pharmacologically stable because the 4-MAA maintains the same pharmacological activity as the MTZ.

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