Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
35 (2004) 479–487
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Characterization of the role of physicochemical factors on the hydrolysis of dipyrone

Hakan Ergün [a,b,*], Daniel A.C. Frattarelli [a], Jacob V. Aranda [a]

[a] *Division of Clinical Pharmacology/Toxicology, Children's Hospital of Michigan, Wayne State University, Detroit, USA*
[b] *Department of Pharmacology and Clinical Pharmacology, Medical School of Ankara University, Merkez Binasi, Sihhiye, 06100 Ankara, Turkey*

Received 15 October 2003; received in revised form 5 February 2004; accepted 6 February 2004

Available online 21 March 2004

Abstract

Dipyrone is a prodrug which is used mainly for its analgesic and antipyretic effects. After oral intake, dipyrone is rapidly hydrolyzed to its main metabolite, 4-methylaminoantipyrine (4-MAA), from which many other metabolites are produced by enzymatic reactions. Even though it is well known that dipyrone is a prodrug and hydrolyzed non-enzymatically, in most of the studies of dipyrone the prodrug form is tested using in vitro methodologies, which do not represent or predict the actual in vivo activity of dipyrone. In this study, we characterize the hydrolysis kinetics of dipyrone as functions of concentration, temperature, and pH using a HPLC assay.

Concentration is an important factor in the hydrolysis of dipyrone. Low concentrations of dipyrone are hydrolyzed more rapidly than are solutions of higher concentrations. At a concentration of 0.1 M, which is 140 times, the concentration of the marketed pharmaceutical form, dipyrone is only minimally (10%) hydrolyzed to 4-MAA at 5 h. Temperature, as expected, affects the hydrolysis reaction dramatically. We tested three temperatures (4, 21, and 37 °C) and found that at body temperature the hydrolysis is significantly faster than at room or at refrigerator temperatures. Compared with more alkaline solutions, the hydrolysis rate of dipyrone increases dramatically in acidic solutions. At low pH (2.5) and at a 0.01 mM concentration, the hydrolysis of dipyrone is completed within almost 30 min, which is the highest rate we observed. Experiments which involve in vitro and/or local application of dipyrone should consider these physicochemical factors and interpret the results accordingly.

Keywords: Dipyrone; Hydrolysis; HPLC; 4-Methylaminoantipyrine

1. Introduction

Although it is well known that physicochemical factors affect chemical compounds in many ways (including changes in their structure as well as in their activity), in daily practice little attention is paid to these. Dipyrone is a typical example of a compound which is affected by these factors.

Dipyrone (also known as metamizol) is a non-narcotic analgesic and antipyretic drug, which is used in both pediatric and adult patients [1,2]. It was introduced into clinical practice in 1922 and is still in use in many countries. It is banned in the USA and Sweden because of a potential side effect, agranulocytosis.

* Corresponding author. Tel.: +90-312-309-52-93; fax: +90-312-311-64-95.
E-mail address: hergun@medicine.ankara.edu.tr (H. Ergün).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.02.004

However, this issue has been criticized by many other authors [3–6]. Due to its strong analgesic effect, available parenteral formulation, and low cost, dipyrone is widely used in Europe (e.g. Germany, Italy, Spain) and South America, and is even an over the counter drug in Turkey and Brazil [7]. Its spasmolytic action along with its analgesic effect makes dipyrone a favorable drug for colic pain [8–12]. Recently, additional beneficial effects of dipyrone (vascular smooth muscle relaxant, antiapoptotic, and anticonvulsant) have been reported and increased the interest in this compound [13–15].

Dipyrone is a prodrug. After oral intake, it is spontaneously hydrolyzed in the gastric fluid to its main metabolite, 4-methylaminoantipyrine (4-MAA), and is absorbed in this form. 4-MAA is then converted to a variety of metabolites by various enzymatic reactions, 15 of which have been identified [16,17]. Dipyrone is briefly detectable in the serum for about 15 min following intravenous administration. It is not detectable after oral intake [17]. The active compound(s) responsible for its effects are not identified, except by their analgesic activity. The onset and duration of the analgesic effect are found to correlate with saliva concentrations of 4-MAA and 4-aminoantipyrine (4-AA) [18]. The contributions of the other metabolites to the effects of dipyrone treatment are poorly understood.

In spite of the knowledge that dipyrone is a prodrug that is not detectable in serum following oral intake (the most widely used route of administration for this drug), most of the in vitro studies measure the concentration of dipyrone itself to analyze and correlate its effects [19–21]. Consequently, the results obtained from the direct quantification of the prodrug dipyrone (as opposed to measurement of its active metabolites) do not reflect the actual benefit of the therapy and therefore, confuse the comparison of in vitro results with clinical observations. In some in vivo animal studies in which dipyrone was given as a local injection, the hydrolysis of dipyrone to 4-MAA was uncertain as well [22,23].

In this study, we aimed to characterize the effects of the physicochemical factors of concentration, temperature, and pH on the hydrolysis of dipyrone to its main metabolite, 4-MAA, and provide data which will guide authors in further experiments to use dipyrone solutions in appropriate way.

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical reagent grade or higher from commercial suppliers. Acetonitrile and methanol were purchased from Merck Chemicals and potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium acetate and dipyrone was purchased from Sigma. Metabolites, 4-MAA, 1,2-dihydro-1,5-dimethyl-4-di-acethylamino-2-phenyl-3H-pyrazol-3-one (4-AAA), 4-formylamino-1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one (4-FAA), and 4-amino-1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one (4-AA) were kindly obtained from Hoechst Marion Roussel (Aventis).

2.2. HPLC system

The HPLC system consisted of an ESA (Bedford, MA) 420 pump having a Rheodyne injector with a 50 μl sample loop and a Perkin-Elmer (Norwalk, MA) T85A UV-Vis detector coupled with a Spectra Physics (San Jose, CA) data jet integrator.

2.3. Chromatographic conditions

Chromatographic separation was achieved on a 3.9 mm × 150 mm SymmetryShield^{TM} RP_8 column (Waters Corp., Massachusetts, USA). The mobile phase was 86 parts of 50 mM sodium acetate buffer pH 6.2 (adjusted using acetic acid) and 14 parts acetonitrile (v/v). Prior to use, the mobile phase was filtered through a nylon filter (pore size: 0.22 mm) and degassed.

The flow rate was 1.0 ml/min, and the detector wavelength was set at 265 nm. The data system provided readout of the digitally integrated area under the peaks, determined the retention time, and calculated the response factors for both dipyrone and 4-MAA. All analyses were performed at room temperature (21 °C).

2.4. Preparation procedure

Two solutions were prepared for all experiments, except for the experiments in which the effects of dipyrone concentration were analyzed at 0.1 M. For

these experiments, dipyrone was diluted in water and used right after the preparation. For the rest of the experiments one stock solution of dipyrone and one of 4-MAA were prepared in 0.1 M methanol. These stock solutions were kept at 4 °C and every experiment in this study was done using these stock solutions. Dilutions for the experiments were done in water, which was generated using MILI-Q water purification system (Milipore Corp., USA) and performed right before the experiments. In experiments performed at low pH (2.5 and 4.0) 100 mM potassium dihydrogen phosphate was used. For the high pH (7.4) experiments, 100 mM dipotassium hydrogen phosphate was used. Adjustment of pH was done using phosphoric acid or 0.1 M NaOH.

2.5. Kinetic methods

2.5.1. Standard curve

Aliquots of 4-MAA stock solution were diluted in water in semi-logarithmic order between 10^{-5} and 10^{-7} M concentrations and analyzed with HPLC to obtain a standard curve. Every morning before the experiments, validation of the system was done with freshly prepared 4-MAA samples. This validation confirmed the stability of 4-MAA stock solution (0.1 M, in methanol) for the entire study period (3 months). As dipyrone is hydrolyzed rapidly after dilution (especially at low concentration and pH) we injected dipyrone samples from dilutions (10 μM) of stock solution prepared right before the injections. We compared the difference of the area under the curve for dipyrone in stock solution for the entire period of the experiments. These test results showed that both dipyrone and 4-MAA are stable in methanol at a concentration of 0.1 M for at least 3 months.

To discriminate between the degradation products detected with small peaks (especially in the experiments done at 37 °C), three other metabolites of dipyrone (4-AAA, 4-FAA, 4-AA) were also assayed with HPLC under the same conditions.

2.5.2. Role of concentration

Aliquots of dipyrone stock solution were diluted to concentrations ranging from 10^{-3} to 10^{-5} M. Concentrations greater than 10^{-5} M were diluted to 10^{-5} M in ice cold water just before injection to decrease any additional hydrolysis which might occur during the time to injection. Samples were kept at room temperature (21 °C) and injected at 0, 15, 30, 45, 60, 90, 120, 180, 240, and 300 min. If the dipyrone peak was not observed, the later time points were not performed. pH and temperature were kept at 4.0 and 21 °C, respectively, for all of the experiments examining the role of concentration. The pH of 4.0 and temperature of 21 °C were chosen to differentiate the effect of concentration more clearly, since at room temperature and pH 4.0 the hydrolysis kinetics were fast enough to be observed during the 300 min period but not so fast as to be completely hydrolyzed before measurement as might be the case at a lower pH or at a higher temperature.

2.5.3. Role of temperature

Two concentrations (10^{-3} and 10^{-5} M) at three temperatures representing refrigerator (4 °C), room (21 °C), and body temperature (37 °C) were used to study the effect of temperature on dipyrone hydrolysis. Samples were kept at pH 7.4 and injected at 0, 15, 30, 45, 60, 90, 120, 180, 240, and 300 min.

2.5.4. Role of pH

Dipyrone was set at 10^{-5} M concentration and three pH values (2.5, 4.0, and 7.4) were used to study the effect of pH. Samples were kept at 21 °C and injected at 0, 15, 30, 45, 60, 90, 120, 180, 240, and 300 min.

3. Results

A typical chromatogram of the hydrolysis of a sample of dipyrone to 4-MAA at different time points is given in Fig. 1.

3.1. Effect of concentration on the hydrolysis of dipyrone

We have used a concentration range of 10^{-1} to 10^{-5} M to observe the effect of concentration on hydrolysis. The percentage change of dipyrone to 4-MAA at seven different concentrations is shown in Fig. 2. Concentration seems to be a major factor affecting the hydrolysis of dipyrone when temperature and pH are kept constant.

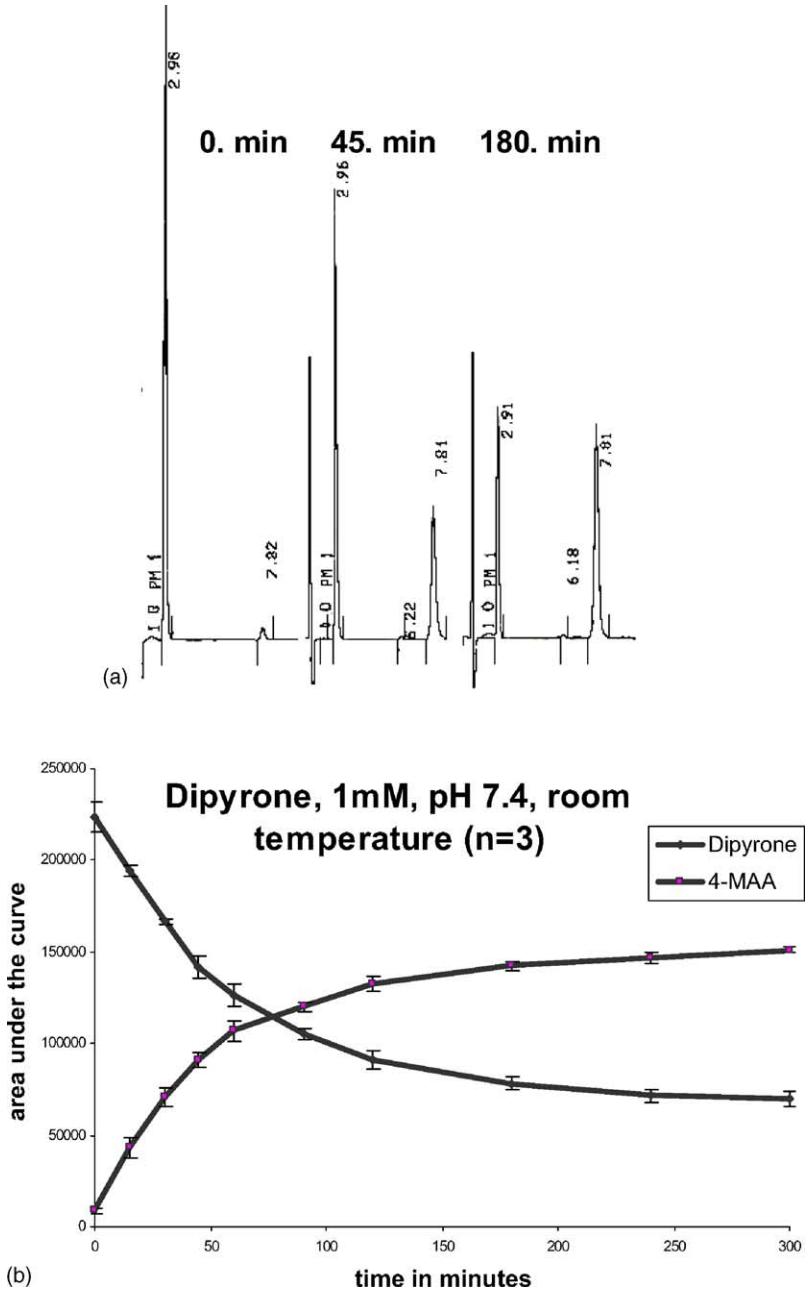

Fig. 1. (a) Typical continuous chromatograms of the hydrolysis from dipyrone (retention time 2.9 min) to 4-MAA (retention time 7.8 min). From left to right 0, 45, and 180 min of incubation at pH 7.4, temperature 21 °C, and concentration of 10^{-3} M and (b) graphical presentation of the hydrolysis of dipyrone. Data are presented as values of peak area under the curve for dipyrone and 4-MAA in the same solution.

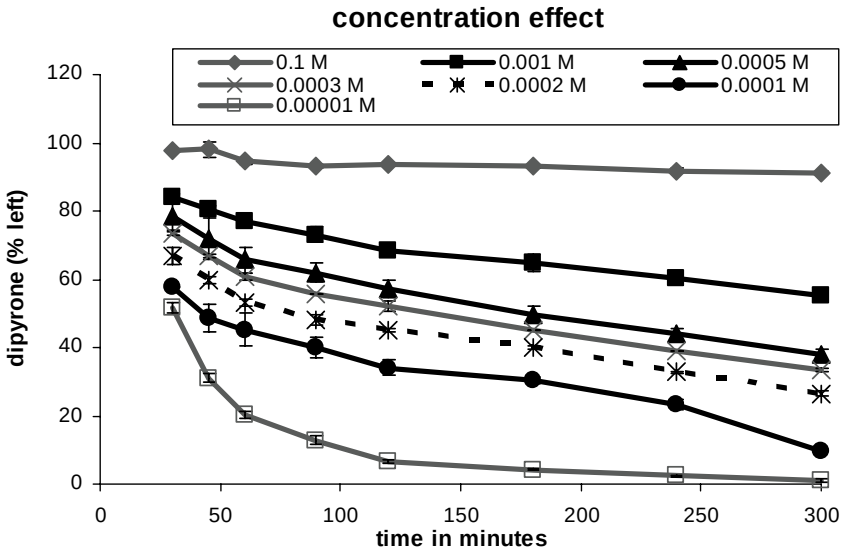

Fig. 2. Concentration effect on the hydrolysis of dipyrone to 4-MAA. Data are presented as percentage change of dipyrone peak area under the curve values from the first time point ($t = 0$) at different concentrations of dipyrone.

3.2. Role of temperature

We have chosen three temperatures (representing refrigerator, room, and body temperatures) and tested at two concentrations (10^{-3} or 10^{-5} M). At constant pH (7.4) and concentration (10^{-3} and 10^{-5} M), temperature increases the rate of the hydrolysis reaction (Fig. 3).

3.3. Role of pH

To evaluate the effect of pH on dipyrone hydrolysis we used a stable temperature (21 °C) and concentration (10^{-5} M). The rate of hydrolysis is more affected by pH than by concentration or temperature, suggesting that acid-catalyzed hydrolysis is involved in the hydrolysis of dipyrone. The effect of pH was linear (Fig. 4).

4. Discussion

In this study, we report the role of concentration, temperature, and pH on the hydrolysis of dipyrone to 4-MAA.

The first factor examined was concentration, which was found to be one of the main factors affecting the spontaneous hydrolysis of dipyrone. Since dipyrone is available as a parenteral formulation and is dissolved in pure water without any preservative, it is interesting to note that dipyrone has a shelf life of nearly 5 years at room temperature. This commercially available solution has a concentration of 1.42 M. We studied a concentration range from 10^{-1} to 10^{-5} M, which includes the plasma 4-MAA levels observed in humans. Concentration is the major factor in the hydrolysis of dipyrone and increasing concentrations of dipyrone decreases the rate of hydrolysis. According to our results, the prolonged stability of dipyrone's commercial formulations (5 years at a concentration of 1.42 M) can be explained by its high concentration.

In most in vitro studies, dipyrone is used at a concentration range similar to our screened concentration range (1 mM–10 μM). From our results we can speculate that in such experiments dipyrone may not be completely hydrolyzed to 4-MAA, and that the degree of hydrolysis will depend upon the time from the preparation of the solution to the analysis of the compound. Because of this ambiguity surrounding the degree of hydrolysis in a given solution, it is not possible to correlate a detected effect with the supposed concentration of dipyrone. The same consideration holds true for in vivo studies in which dipyrone is applied locally and its effect is looked for immediately, before

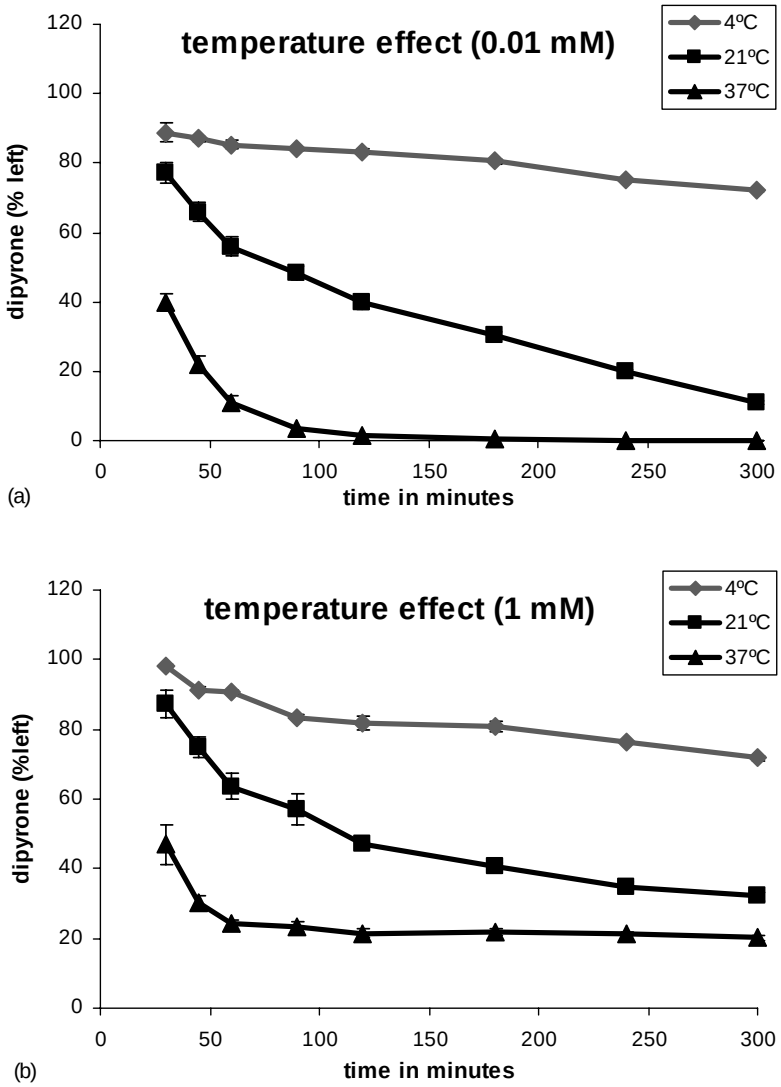

Fig. 3. Effect of temperature on the hydrolysis of dipyrone. Graphics represent the percentage change of dipyrone peak area under curve values from the first time point ($t = 0$) at different temperatures (4, 21, and 37 °C) and concentrations (a) 0.01 mM and (b) 1 mM of dipyrone.

dipyrone has been hydrolyzed at the local tissue level or absorbed into the systemic circulation and returned as an active metabolite. In such in vivo experiments, it is important to measure the levels at the local site for both of the parent drug and its metabolite(s) to draw a meaningful correlation with its effects.

Moreover, it is very important to notice that in clinical usage 4-MAA is absorbed into the systemic circulation and all of the supposed effects of dipyrone are related to 4-MAA or its other active metabolites. There is only one exception: when dipyrone is administered intravenously it is detectable in plasma for a short period of time, but none of its effects are seen within this time period [17].

Temperature is always an important factor in drug disposition. We have studied the hydrolysis of dipyrone at three temperatures commonly encountered in practice: refrigerator, room temperature, and body temperature. As predicted thermodynamically, higher temperatures add energy to the system and can

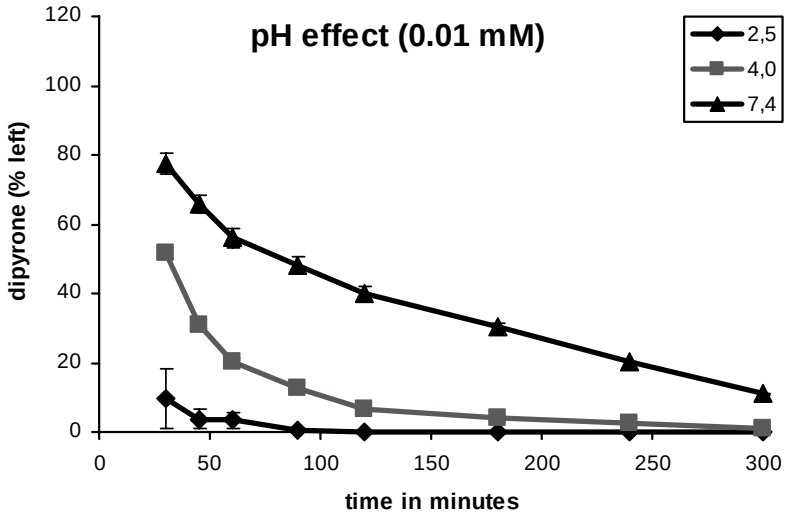

Fig. 4. Effect of pH on the hydrolysis of dipyrone. Graphics represent the percentage change of area under dipyrone peak at different pH (2.5, 4.0, and 7.4) and 0.01 mM concentration of dipyrone.

increase the rate of catalysis of the reaction. To see if temperature itself can increase the rate of hydrolysis at higher concentrations of dipyrone, we used a 0.1 M solution at 4 and 37 °C and found that at this concentration this increase in temperature was not associated with acceleration of the reaction. This demonstrates that even at body temperature the hydrolysis of dipyrone to its main metabolite will not be increased, which is an important consideration for local administrations of dipyrone. In clinical pharmacokinetic trials, it has been shown that intramuscular administration of dipyrone has almost the same pharmacokinetic profile as oral administration and that the t_{max} value for intramuscular injection is about 1.7 h (bioavailability 87%) [17]. It may be speculated that the administered dipyrone is absorbed slowly, and at the local injection site dipyrone itself is present at a greater concentration than is its metabolite 4-MAA.

According to our data, when dipyrone is applied to biological tissues in in vivo or in vitro experiments, the efficacy, if measured before 5 h, will not accurately represent the actual activity of either dipyrone or 4-MAA, as it is not possible to discriminate which of the compounds is responsible. In clinical practice, dipyrone is administered mainly by the oral route. After oral administration the drug it is rapidly hydrolyzed to 4-MAA and is absorbed in that form. There is no observable dipyrone level in plasma. However, when dipyrone is administered intravenously, it can be detected in the serum for 15 min. Since the effects of dipyrone may be achieved by either of these administration routes, the active compound has to be one of its metabolite and not the dipyrone itself. The actual effects are produced by 4-MAA and probably by other metabolites as well. There is only one study in which the correlation of the metabolites' serum concentration and efficacy has been described [18]. In this study, 4-AA and 4-MAA were found to correlate with the analgesic effect of dipyrone. However, there are no data about the relation of other metabolites of dipyrone and its other effects (i.e. antipyretic, antispasmotic).

Another interesting point is the rare but sometimes serious hypotensive side effect of dipyrone [24,25]. Although it has not been systematically studied, there is anecdotal evidence about this effect, which may be observed after the intravenous administration of dipyrone. This effect is not well studied and there is not much information about its mechanism. In our previous clinical efficacy trial using dipyrone to treat urinary colic pain we monitored the blood pressure of the subjects. We did not find any significant effect on blood pressure after 1 g of dipyrone was administered intramuscularly. Similarly, other clinical efficacy trials involving dipyrone did not report any decrease in blood pressure after intramuscular or oral administration [26,27]. This administration route dependent

hypotensive side effect of dipyrone may be explained by the differences in the degree of hydrolysis between administration routes and the consequent presence of the main compound (dipyrone) in systemic compartment after intravenous administration in contrast to other routes of administration.

The third and the last factor we studied was the pH. We found that more acidic pH dramatically increased the rate of hydrolysis. At a low concentration of dipyrone (10^{-5} M), the rate of hydrolysis versus pH was linear. A pH of 2.5 was the most acidic solution used in our experiments and was associated with the highest rate of hydrolysis of dipyrone. The results from our study suggest that acid-catalyzed hydrolysis is the main mechanism for the hydrolysis of dipyrone. We did not study possible other mechanisms such as autocatalysis or self-inhibition to identify other factors which may explain our findings and which need to be examined in further studies.

From the results obtained in this study, we conclude that in patients with normal gastric fluid pH levels and body temperature, oral dipyrone is totally converted to 4-MAA before its passage to duodenum. One may wonder why then the prodrug dipyrone is administered and not just the 4-MAA, the active compound to which it is metabolized. It is not because of an increase in bioavailability, as it is occasionally the case with other medications. The oral bioavailability of dipyrone is almost complete (85%) [17]. Sometimes, a prodrug is used instead of the active compound if there is a problem with the solubility of the active metabolite. Yet to the best of our knowledge and from our experience in this study, no solubility problems with 4-MAA were noted at concentrations of 0.1 M and lower. Another reason for the use of a prodrug is to obtain a better stability than the active metabolite; this seems to be the case with dipyrone and 4-MAA.

We have found that at body temperature, the production of two compounds, one with the same retention time of 4-AA (6.2 min) and the other with a retention time of 5.7 min, increases over the first 1–2 h and then stabilizes for the next 24 h. For a 5 h observation period, we did not observe any correlation between the hydrolysis to 4-MAA ratio and the observable peak with the compounds detected. In overnight incubation at 37 °C, the number of the compounds detected with HPLC increases, indicating that 4-MAA is not stable under these conditions when dissolved in water. However, the dilution of 4-MAA in methanol was stable for the entire time period of our experiments (3 months). Additionally, the amount of 4-MAA significantly decreased overnight at both room and body temperature, while the production of the other compounds with similar retention times was almost stable.

As we did not aim to identify the chemical structure of the compounds, we cannot confirm with certainty that one of the degradation products of 4-MAA is 4-AA, as both have the same retention time in our HPLC system. Further studies to identify these degradation products should be performed with crystallography and other appropriate methods.

In this study, we have shown that the prodrug dipyrone is rapidly hydrolyzed to its active metabolite at physiologic temperature, concentration, and pH. Future studies measuring the effects of dipyrone should, therefore, not focus on concentrations of dipyrone, but rather on the concentrations of one or more of its metabolites. Further research is necessary to elucidate the identity and role of these metabolites in the effects of dipyrone therapy. Investigators should also be aware of its spontaneous hydrolysis in the refrigerator and on the benchtop under certain conditions, and should design their experiments accordingly.

Acknowledgements

Dr. Hakan Ergün is sponsored partially by TUBITAK (NATO-B2). Drs. Ergün and Frattarelli are sponsored by Children's Research Center of Michigan and the NIH Pediatric Pharmacology Research Unit at Wayne State University.

References

[1] R.N. Brogden, Drugs 32 (Suppl. 4) (1986) 60–70.
[2] A. Wong, A. Sibbald, F. Ferrero, M. Plager, M.E. Santolaya, A.M. Escobar, S. Campos, S. Barragan, M. De Leon Gonzalez, G.L. Kesselring, Clin. Pediatr. (Phil.) 40 (2001) 313–324.
[3] The international agranulocytosis and aplastic anemia study, JAMA 256 (1986) 1749–1757.
[4] M. Bensenor, Sao Paulo Med. J. 119 (2001) 190–191.
[5] C. Berlin, Clin. Pediatr. (Phil.) 40 (2001) 325–326.
[6] J.E. Edwards, H.J. Edwards, Lancet 360 (2002) 1438.

[7] S. Grond, L. Radbruch, T. Meuser, R. Sabatowski, G. Loick, K.A. Lehmann, Pain 79 (1999) 15–20.
[8] G. Stankov, G. Schmieder, G. Zerle, S. Schinzel, K. Brune, World J. Urol. 12 (1994) 155–161.
[9] G. Brandstatter, S. Schinzel, H. Wurzer, Dig. Dis. Sci. 41 (1996) 1814–1818.
[10] J.E. Edwards, F. Meseguer, C. Faura, R.A. Moore, H.J. McQuay, Cochrane Database Syst. Rev. 4 CD003867 (2002).
[11] C. Muriel-Villoria, E. Zungri-Telo, M. Diaz-Curiel, M. Fernandez-Guerrero, J. Moreno, J. Puerta, P. Ortiz, Eur. J. Clin. Pharmacol. 48 (1995) 103–107.
[12] H.J. Schroth, H. Garth, S. Rupp, A. Steinstrasser, Fortschr. Med. 104 (1986) 378–382.
[13] H. Ergun, İ.T. Uzbay, T. Celik, H. Kayir, Ö. Yesilyurt, F.C. Tulunay, Drug Dev. Res. 53 (2001) 254–259.
[14] C. Pompeia, M.F. Boaventura, R. Curi, Int. Immunopharmacol. 1 (2001) 2173–2182.
[15] H. Ergun, I.H. Ayhan, F.C. Tulunay, Gen. Pharmacol. 33 (1999) 237–241.
[16] E. Zylber-Katz, L. Granit, M. Levy, Eur. J. Clin. Pharmacol. 42 (1992) 187–191.
[17] M. Levy, E. Zylber-Katz, B. Rosenkranz, Clin. Pharmacokinet. 28 (1995) 216–234.
[18] P. Rohdewald, G. Drehsen, E. Milsmann, H. Derendorf, Arzneimittelforschung 33 (1983) 985–988.
[19] G. Galati, S. Tafazoli, O. Sabzevari, T.S. Chan, P.J. O'Brien, Chem. Biol. Interact. 142 (2002) 25–41.
[20] C. Campos, R. de Gregorio, R. Garcia-Nieto, F. Gago, P. Ortiz, S. Alemany, Eur. J. Pharmacol. 378 (1999) 339–347.
[21] A.A. Pigoso, S.A. Uyemura, A.C. Santos, T. Rodrigues, F.E. Mingatto, C. Curti, Int. J. Biochem. Cell Biol. 30 (1998) 961–965.
[22] D. Alves, I. Duarte, Eur. J. Pharmacol. 444 (2002) 47–52.
[23] N. Hernandez, H. Vanegas, Brain Res. 896 (2001) 175–178.
[24] M. Zoppi, R. Hoigne, M.F. Keller, F. Streit, T. Hess, Schweiz Med. Wochenschr. 113 (1983) 1768–1770.
[25] R. Hoigne, M. Zoppi, J. Sollberger, T. Hess, D. Fritschy, Agents Actions Suppl. 19 (1986) 189–195.
[26] R. Babej-Dölle, S. Freytag, J. Eckmayer, G. Zerle, S. Schinzel, G. Schmieder, G. Stankov, Int. J. Clin. Pharmacol. Ther. 32 (1994) 204–209.
[27] H.E. Romero, A.C. Fernandez, T.C. Rosales, Arzneimittelforschung 26 (1976) 738–740.