ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
26 (2001) 1003–1008

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Short communication

LC determination of dinitrosopiperazine in simulated gastric juice

M.I. Walash *, F. Belal, F. Ibrahim, M. Hefnawy, M. Eid

Department of Analytical Chemistry, Faculty of Pharmacy, University of Mansoura, Mansoura, 35516, Egypt

Received 10 April 2001; accepted 15 April 2001

Abstract

A simple and specific reversed phase HPLC method for the determination of dinitrosopiperazine in simulated gastric juice using UV detection was reported. The chromatographic resolution of the analyte and the internal standard isosorbide dinitrate was performed without extraction from the gastric juice on a reversed phase ODS column. Isocratic elution was carried out with methanol–0.02 M sodium dihydrogen phosphate (60:40 v/v, pH 3.0) at a flow rate of 1.0 ml min^{-1} with UV detection at 238 nm. The calibration graph was linear over the concentration range 0.072–2.88 μg ml^{-1} of dinitrosopiperazine with minimum detectability ($S/N = 2$) of 0.01 μg ml^{-1} (8×10^{-8} M). Inter-day and intra-day precisions calculated as% RSD were in the range 0.32–0.38% and 0.19–0.25% respectively. Inter-day and intra-day accuracies calculated as% error were in the range 0.18–0.21 and 0.08–0.11% respectively. The proposed method was successfully applied to the study of the possible in–vivo production of DNPZ under the standard nitrosation conditions recommended by WHO.

Keywords: Gastric juice; Dinitrosopiperazine (DNPZ); Piperazine; HPLC

1. Introduction

N–nitroso compounds are powerful carcinogens that are formed when amines and amides react with nitrite [1]. Their formation has been demonstrated in gastric juice of experimental animals [2,3] and humans [4,5]. Gastric cancer is more common in areas where drinking water contains a lot of nitrate and bacteria which reduce nitrate to nitrite [6,7]. Drugs with amino or amide groups have been shown to yield genotoxic *N*–nitroso derivatives [8–10].

Piperazine is widely used as an anthelmintic drug, in addition to being active in the treatment of gout and urinary lithiasis by increasing uric acid excretion and dissolving urate crystals [11]. It is rapidly nitrosated under simulated gastric juice (SGJ) conditions to produce the carcinogens *N*–mononitrosopiperazine (MNPZ) and *N*,*N*–dinitrosopiperazine (DNPZ) [12]. DNPZ–being the

* Corresponding author. Fax: +20-50-247496.
E-mail address: manaleid@mum.mans.eun.eg (M.I. Walash).

0731-7085/01/$ - see front matter
PII: S0731-7085(01)00509-X

more potent carcinogen [13] is not immediately formed, however MNPZ is probably—in part—converted by transnitrosation to DNPZ [14]. Long term, low-grade exposure to piperazine may also involve a considerable risk, not least since the more potent carcinogen DNPZ is more likely to be produced when nitrite is in excess of piperazine [12]. These findings support the views that piperazine drugs should be formulated with a nitrite trap (e.g. ascorbic acid) to slow down the nitrosation reaction. For *N*–nitrosopiperazine in biological fluids the literature revealed that, it has been determined by spectrophotometery [15,16], voltammetry [17], TLC [18] and GLC using nitrogen–specific flame ionization detection [19], or with thermal energy analysis [20]. These GLC methods offer a high degree of specificity sample clean-up and instrument limitations preclude their use in routine clinical studies. Therefore there is still a need for an alternative substitute for these methods. A detailed overview in the literature revealed that no study has yet determined the possible in-vivo production of DNPZ in SGJ using HPLC method.

HPLC is the reliable substitute used in checking the possible formation of *N*–nitroso compounds and directly calculating their amounts in the reaction mixture with higher degree of accuracy. This paper describes a reversed phase HPLC method using UV-detection to measure low concentrations of *N*, *N*–dinitrosopiperazine in gastric juice. The method is linear over the range 0.072–2.88 μg ml^{-1} with detection limit of 0.011 μg ml^{-1} ($S/N = 2$).

2. Experimental

2.1. Materials and reagents

Piperazine citrate was obtained from Pharco Pharmaceutical Co. (Alexandria, Egypt). *N*, *N*–dinitrosopiperazine was synthesized in our laboratory according to the method described by USP 24 [21] and it is stable for months in its dry form. lsosorbide dinitrate was obtained from EIPICO Pharmaceutical Co (Cairo, Egypt). Pepsin was obtained from Sigma Chimica (Milan, Italy). Simulated gastric juice (SGJ) was prepared according to USP 24 [21]. Methanol (Merck) was HPLC grade. Sodium dihydrogen phosphate (0.02 M) was prepared. The mobile phase was isocratic mix. of methanol–0.02 M sodium dihydrogen phosphate (60:40 v/v) was prepared and adjusted to pH 3.0 by phosphoric acid. All chromatographic solutions were filtered through 0.45 μm memberane filter (Gilman Instrument Co.).

2.2. Chromatographic conditions and instrumentation

The chromatographic analyses were performed with a Merck Hitachi Chromatograph L-7100, injector valve with a 20 μl loop and a Merck Hitachi L-7400 UV detector (Darmstadt, Germany). Retention times, peak areas and UV spectra were recorded on a Merck Hitachi D-7500 integrator. Solvents were degassed using Merck Solvent L–7612 degasser. A Hibar® pre- packed column RT 250-4, Lichrosorb® RP-18 (5 μm) combined with guard column (Darmstadt, Germany) was used.

2.3. Standard solutions

Standard solution of *N*,*N*-dinitrosopiperazine was prepared by dissolving 36.0 mg in 15.0 ml of methanol,then completing to 25.0 ml with SGJ in a measuring flask. This solution was further diluted with the mobile phase to give the final concentration required for preparation of calibration graph. The solution is stable for at least one week when kept in the refrigerator.

2.4. Procedure for calibration curve

Transfer aliquots of the standard solution containing DNPZ within the concentration range cited in Table 1 into a 10 ml measuring flask, add 0.5 ml of 1.0 mg ml^{-1} of the internal standard (I.S.) solution. Complete to the mark with the mobile phase. A 20 μl aliquot was injected (triplicate) and the calibration curve was constructed by plotting the peak area ratios against the final concentration of DNPZ. Unknown concentration of DNPZ was quantified by relating the respective

peak area ratios to the regression line; data are presented as percentage of DNPZ.

2.5. Conditions of nitrosation

To check the possible formation of DNPZ from the interaction between the drug and the nitrite-rich gastric juice, the nitrosation reactions were carried out under the conditions recommended by WHO [22] (condition A), in physiological like conditions containing a therapeutic dose of the drug (condition B) and within the limit of quantitation of the method (condition C). All the reactions were carried out at 37 °C with stirring for 3 h and checked at 5, 15, 30 and up to 180 min.

2.5.1. Condition A

In this reaction the following concentrations were used: drug, 10m M l^{-1}; and sodium nitrite, 40 mM l^{-1} at pH 1.2 and 3.5 in SGJ and in distilled water.

2.5.2. Condition B

The following concentrations were used: drug, 2 m M l^{-1}; and sodium nitrite, 2.9 mM l^{-1} at pH 1.2 and 3.5 in SGJ and in distilled water.

2.5.3. Condition C

The following concentrations were used: drug, 2 mM l^{-1}; and sodium nitrite, 0.2 mM l^{-1} at pH of 1.2 and 3.5 in SGJ and in distilled water.

Table 1
Analysis of standard *N*,*N*-dintrosopiperazine by the proposed HPLC method in simulated gastric juice

Taken μg	Found μg	Recovery
0.072	0.0725	100.69
0.144	0.1447	100.48
0.288	0.2868	99.58
0.720	0.7202	100.02
1.440	1.4429	100.20
2.880	2.8823	100.08
$X \pm$ S.D.		100.17 ± 0.38
R.S.D.%		0.37
Er%		0.15
S_a		9.04×10^{-4}
S_b		2.62×10^{-4}

3. Results and discussion

3.1. Method development

A simple and reliable HPLC method was developed for the determination of DNPZ in simulated gastric juice. To validate and optimize the method, different mobile phases were used in order to achieve the best separation and resolution of the eluted peaks in SGJ. The effect of organic modifier was studied using acetonitrile or methanol with different concentrations of sodium dihydrogen phosphate. The separation and resolution of the peaks ($Rs = 3.24$) could be achieved upon using mixture of sodium dihydrogen phosphate (0.02 M) and methanol in the ratio 40:60 v/v. Both methanol and acetonitrile were equally useful as an organic modifier. The pH of sodium dihydrogen phosphate solution was also studied by changing the pH over the range 3.0–7.0, the optimum separation was accomplished upon using solution of pH 3.0 ± 0.2. Solutions of lower pH values were not attempted to guard against the deterioration of the stationary phase. Different ionic strengths of sodium dihydrogen phosphate (0.0075–0.06 M) were studied, the optimum resolution was obtained upon using 0.02 M solution of sodium dihydrogen phosphate.

3.2. Characteristics of the chromatographic peaks

According to the conditions described, the retention times were about 1.6, 2.40 and 4.60 min for SGJ alone, DNPZ and I.S. respectively. Typical chromatogram of DNPZ after reaction between piperazine and sodium nitrite in SGJ is shown in Fig. 1. The retention time was suitable enough to allow determination of DNPZ without interference from the SGJ.

3.3. Validation

3.3.1. Linearity

The linearity for DNPZ was assessed over the range 0.072–2.88 μg ml^{-1}. Varying volumes of standard solution were transferred into a 10 ml volumetric flasks to give concentrations cited in Table 1 then 0.5 ml of I.S. solution was added to

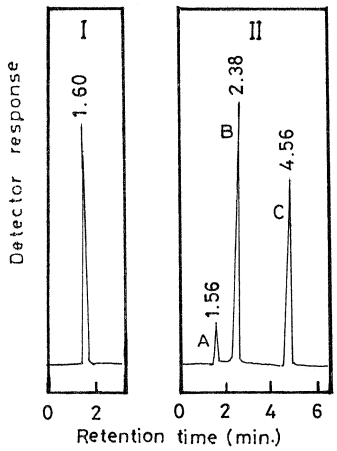

Fig. 1. Typical chromatograms of (I) drug-free SGJ and (II) SGJ with dinitrosopiperazine (0.8 μg ml^{-1}, 2.38 min) and internal standard isosorbide dinitrate (50 μg ml^{-1}, 4.56 min).

each flask. Complete to the mark with the mobile phase. Triplicate injections were made at each concentration. The linearity of the standard curve was confirmed by plotting the ratio of the DNPZ to I.S. peak areas versus the concentration of DNPZ. A straight line obtained in the range of 0.072–2.88 μg ml^{-1}. Alternatively, the following regression equation was described $Y = 0.0366 + 0.335\ C$, $r = 0.9999$, where Y = peak area ratio of DNPZ to I.S. and C = concentration of DNPZ in μg ml^{-1}.

3.3.2. Limits of quantitation (LOQ) and limit of detection (LOD)

The limit of quantitation (LOQ) was determined by establishing the lowest concentration that can be measured with acceptable accuracy and precision; in this case DNPZ can be quantified under these conditions at concentration of 0.072 μg ml^{-1}. The limit of detection (LOD) was determined by establishing the minimum level at which the analyte can be reliably detected and it was found to be 0.01 μg ml^{-1} based on a signal-to-noise ratio of 2.

3.3.3. Accuracy and precision

The intra-day and inter-day precision and accuracy data are summarized in Table 2. The repeatability of the assay was found to be within 0.19–0.24% ($n = 5$) at 0.8 and 1.6 μg ml^{-1}. The reproducibility of the assay at the same concentration levels was found to be within 0.32–0.37%. The intra-day and inter-day accuracy calculated as Er% was within 0.08–0.11 and 0.18–0.21% ($n = 3$), respectively.

3.3.4. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters. To optimize the assay parameters, the effect of pH, organic modifier and ionic strength on the capacity factor (K) values were studied. Although this capacity factor was maintained 0.62–0.66 at pH range 3–5, a pH of 3 ± 0.2 was selected as it has the highest number of theoretical plates (N).

Table 2
Accuracy and precision data for *N*,*N*-dinitrosopiperazine in simulated gastric juice

	Conc. added (μg ml^{-1})	Conc. found (μg ml^{-1})			
		Mean ± S.D.[a]	% Recovery	R.S.D.%[b]	Er%[c]
Intra-day	0.8	0.815 ± 0.002	101.92 ± 0.25	0.24	0.11
	1.6	1.595 ± 0.003	99.71 ± 0.19	0.08	0.08
Inter-day	0.8	0.814 ± 0.003	101.83 ± 0.38	0.37	0.21
	1.6	1.594 ± 0.005	99.64 ± 0.32	0.32	0.18

[a] Mean and S.D. ($n = 5$ for intra-day, $n = 3$ for inter-day).
[b] Relative S.D.
[c] Percentage relative error.

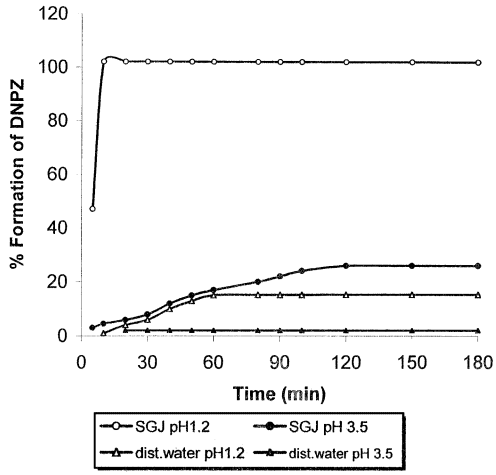

Fig. 2. Formation of DNPZ (under condition A).

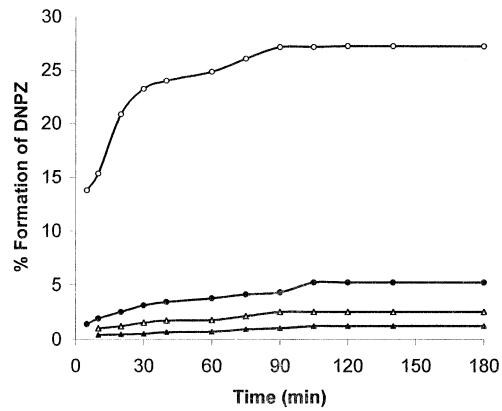

Fig. 3. Formation of DNPZ (under condition B).

3.4. Application

Establishing the conditions of the proposed method, it was applied to follow-up the nitrosation reaction of piperazine in SGJ at pH values of 1.2 and 3.5 under the conditions recommended by WHO, in physiological like conditions containing a therapeutic dose of the drug and within the limit of quantitation of the method. The yields of DNPZ were determined after 5, 10, 15 and up to 180 min, so that the time course of the nitrosation reaction was examined at an interval which is longer than the normal emptying times of the stomach, taking into account some pharmacological and pathological conditions which may delay actual stomach emptying. The formation of DNPZ was found to be immediately after the addition of sodium nitrite to the drug in SGJ and the amount of DNPZ increased quickly during the first 20 min at pH 1.2 then became constant up to the third hour. While at pH 3.5 its formation continues up to 2 h then became constant as shown in Figs. 2–4. The DNPZ yield was higher at pH 1.2 than at pH 3.5 and higher in SGJ than in distilled water in all conditions. The component responsible for this enhancement was pepsin [23].

4. Conclusion

The described HPLC method is simply, reproducible, rapid and has adequate sensitivity to measure dinitrosopiperazine in gastric juice. The method can measure concentration down to 0.072 μg ml^{-1}. The detection limit of 0.011 μg ml^{-1} is comparable to those of the other reported methods. The method was developed as a promising substitute to the reported GC methods. However, the suggested method has some distinct advantages regarding simplicity and reproducibility.

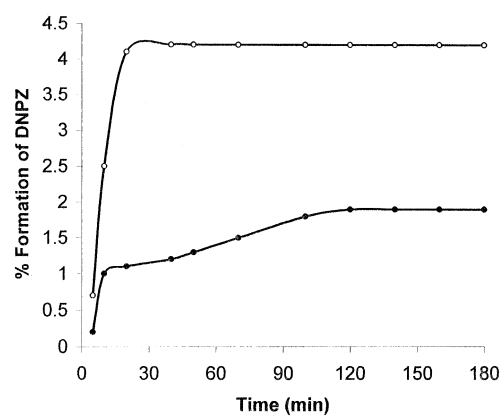

Fig. 4. Formation of DNPZ (under condition C).

References

[1] W. Lijinsky, H.W. Taylor, Cold Spring Harbor Lab. 4 (1977) 1579.
[2] S.S. Mirvish, J. Natl. Cancer Inst. 71 (1983) 629.
[3] S.S. Hecht, J.B. Morrison, Cancer Res. 44 (1984) 2873.
[4] A. Martelli, A. Allaven, E. Sottofattori, G. Brambilla, Toxicol. Lett. 73 (1994) 185.
[5] I.R. Rowland, T. Granli, O.C. Bockman, P.E. Key, R.C. Massy, Carcinogenesis 12 (1991) 1395.
[6] P. Correa, W. Haenszel, C. Cuello, S.R. Tannenbaum, M. Archer, Lancet II (1975) 58.
[7] M.J. Hill, G. Hawksworth, U. Tattersall, Br. J. Cancer 28 (1973) 562.
[8] G. Brambilla, Pharmacol. Res. Commun. 17 (1985) 307.
[9] B. Bellander, B.G. Osterdahl, L. Hagmar, International Agency for Research on Cancer, vol. 37, IARC, Lyon, 1983, pp. 811–818.
[10] E. Sottofattori, M. Anzalcli, A. Balbi, A. Martelli, Il Farm. 52 (1997) 123.
[11] I.M. Kollo, in: L.S. Goodman, A. Gilman (Eds.), The Pharmacological Basis of Therapeutics, 6th, Macmillan, New York, 1980, p. 1023.
[12] B. Bellander, L. Hagmar, B.G. Osterdahl, Lancet II (1981) 372.
[13] H. Garci, L. Keefer, W. Lijinsky, C.E.M. Wenyon, Z. Krebsforsch 74 (1970) 179.
[14] W. Lijinsky, R.M. Kovatch, Cancer Lett. 74 (1–2) (1993) 101.
[15] D. Ziebarth, Arch. Geschwultforsch 51 (1981) 587.
[16] D. Ziebarth, in: P. Bogovski, E.A. Walker (Eds.), N–nitroso Compounds in the Environment, IARC, Lyon, 1975, p. 137.
[17] F. Belal, M.I. Walash, F. Ibrahim, M. Hefnawy, M. Eid, Il Farm. 55 (2000) 694.
[18] N.P. Sen, B. Donaldson, in: P. Bogovski, E.A. Walker (Eds.), *N*–nitroso Compounds in the Environment, IARC, Lyon, 1975, p. 103.
[19] J. Sander, J. LaBar, M. Ladenstein, F. Schweinsberg, in: P. Bogovski, E.A. Walker (Eds.), *N*-nitroso Compounds in the Environment, IARC, Lyon, 1975, p. 123.
[20] B.G. Osterdahl, B. Bellander, J. Chromatogr. 278 (1983) 71.
[21] United States Pharmacopoeia XXIV. National Formulary 19, Rockville, USP Convention, Washington (2000) p. 1341, 2235.
[22] International Agency for Research on Cancer, IARC, Monographs on the evaluation of the carcinogenic risk of chemicals to humans: some pharmaceutical drugs, Vol. 24, IARC, Lyon, (1980) 297–314.
[23] G. Brambilla, A. Martelli, E. Sottofattori, Carcinogenesis 16 (1995) 1239.