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Cation-exchange high-performance liquid chromatographic assay of piperazine in some pharmaceutical formulations

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Abstract

An assay method for the quality control of piperazine in some formulations was developed utilizing cation-exchange high-performance liquid chromatography. A sample solution, containing 1-phenylpropanolamine–HCl as internal standard, was chromatographed on a 250 × 4.6 mm I.D. Ultrasil CX column with an aqueous mobile phase containing 0.07 *M* KH_2PO_4 (pH 3.0)–triethylamine (100:0.01), and differential refractive index detection. Piperazine and 1-phenylpropanolamine–HCl eluted at about 4.9 and 6.4 min, respectively, with a resolution of 2.1. Piperazine/internal standard peak area ratio was linear over 4–477 μg of piperazine dihydrochloride monohydrate injected ($r = 0.9994$). The limit of quantitation was 5.3 μg of piperazine dihydrochloride monohydrate injected. Recovery studies covering a range of ± 33% of label amount of piperazine in commercial formulations gave an overall recovery (± S.D., $n = 6$) of 100.2 ± 0.8% from spiked tablet placebos, and 100.3 ± 1.0% from spiked syrup placebos. The method was tested to be rugged based on Youden and Steiner's experimental design. The assay results of commercial formulations were higher than those obtained by the USP method. Stability tests indicated that degradation products of piperazine, formed upon hydrogen peroxide treatment, did not interfere with the piperazine peak, whereas piperazine dihydrochloride aqueous solutions were fairly stable in acid, base, and exposure to short-wavelength UV light.

1. Introduction

Piperazine (diethylenediamine), a heterocyclic nitrogenous compound, is an anthelminthic agent. It has been used in the treatment of severe infections due to *A. lumbricoides* and *E. vermicularis* [1]. The commercial formulations are available in the form of tablets, granules, syrups and incorporated into feeds. These formulations usually contain piperazine as either the dihydrochloride, sulfate, or citrate.

Analysts have utilized many methods for the assay of piperazine and its salts. These methods include gravimetric [2–5], colorimetric [6–9], titrimetric [4,10], spectrophotometric [11], polarographic [12] and gas chromatographic methods [13–17].

The official assay methods listed in both USP XXII [18] and British Pharmacopoeia 1988 [19] for piperazine base and piperazine citrate are based on non-aqueous titration with acetous perchloric acid titrant. The non-aqueous titration

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is currently also being proposed as the assay method for piperazine dihydrochloride [20]. Although the non-aqueous titration method gave fairly good results, the use of acetous perchloric acid is not desirable as perchloric acid is a hazardous material. Furthermore, glacial acetic acid is a liquid that fills the room with a pungent odor (even when working in the hood) and produces burns on the skin upon contact. The official assay methods for the dosage forms of piperazine salts (tablets, syrups) are gravimetric in which trinitrophenol (picric acid) is used as precipitating reagent. These methods are time consuming and somehow dangerous, because picrates may explode during the drying of piperazine picrate at 105°C to constant mass. In addition, the method calls for washing the piperazine picrate with absolute ethanol. This step is the source for losses as the picrate redissolves in absolute ethanol that contains traces of moisture. This paper describes a direct and simple HPLC assay method for both piperazine and piperazine dihydrochloride in bulk material and some commercial formulations.

2. Experimental

2.1. Apparatus

The following apparatus were used: a Beckman Model 330 isocratic liquid chromatograph with a Model 110A single-piston reciprocating pump (Beckman Instruments, Fullerton, CA, USA); an Altex Model 210 high-pressure sample injection valve with a 20-μl sample loop; a Model R-401 refractive index detector (Waters Chromatography, Milford, MA, USA), and a Varian 4270 electronic integrator (Varian Instruments, Walnut creek, CA, USA); Chromato-Vue CC-20 (Ultraviolet Products, San Gabriel, USA).

2.2. Reagents and materials

The following reagents were used: anhydrous piperazine (crystalline), piperazine dihydrochloride monohydrate, piperazine citrate tetrahydrate, 1-phenylpropanolamine hydrochloride, picric acid (Sigma, St. Louis, MO, USA); triethylamine, potassium monobasic phosphate (Fisher Scientific, Fair Lawn, NJ, USA); simulated tablet placebo (containing: corn starch, lactose, mannitol, magnesium stearate, calcium phosphate, methyl cellulose, povidone, talcum, FD&C Blue No. 1 lake); simulated syrup placebo (containing tartrazine, methylparaben, sorbitol, water), deionized water (Millipore, Bedford, MA, USA). All were used as received. Other chemicals used were analytical grade.

2.3. HPLC conditions

A 250 × 4.6 mm I.D. 10 μm Ultrasil CX (Beckman Instruments) cation-exchange column was used at ambient temperature with an isocratic aqueous mobile phase containing 0.07 *M* KH_2PO_4 (pH 3.0) buffer–triethylamine (100:0.01) at a flow-rate of 1.0 ml/min. The differential refractometer (24°C) was set at attenuation of 16 × and its reference cell was filled with mobile phase. The electronic integrator was set at an attenuation of 4 and a chart speed of 0.5 cm/min. The mobile phase was filtered through a 0.45-μm nylon-66 membrane filter and degassed prior to use.

2.4. Internal standard solution

About 1.770 g of 1-phenylpropanolamine–HCl was accurately weighed and transferred into a 50-ml volumetric flask, dissolved and diluted to volume with 0.07 *M* KH_2PO_4 buffer (pH 3.0).

2.5. Standard solution preparation

About 55 mg of piperazine dihydrochloride monohydrate (equivalent to about 27 mg of anhydrous piperazine base) was accurately weighed and transferred into a 10-ml volumetric flask. Exactly, 1.0 ml of internal standard solution was added, and the solution was diluted to volume with 0.07 *M* KH_2PO_4 buffer (pH 3.0).

2.6. Sample solution preparation

Tablets

Twenty tablets were weighed accurately and finely pulverized in a mortar. An aliquot, equivalent to 55 mg piperazine dihydrochloride monohydrate, was weighed accurately and transferred into a 10-ml volumetric flask. After addition of 1.0 ml of internal standard solution, the mixture was diluted to volume with 0.07 *M* KH_2PO_4 buffer (pH 3.0), sonicated for 15 s, and filtered. After discarding the first 5 ml, the filtrate was collected and chromatographed.

Syrups

The specific gravity of the syrup was determined following the compendial procedure [21]. An amount of syrup, containing the equivalent of about 275 mg of piperazine dihydrochloride monohydrate (equivalent to about 134 mg of anhydrous piperazine base), was weighed accurately into a 50-ml volumetric flask. After addition of 5.0 ml of the internal standard solution, the mixture was diluted to volume with 0.07 *M* KH_2PO_4 buffer (pH 3.0) buffer and chromatographed. Following chromatography, the amount of piperazine dihydrochloride monohydrate found in mg/g was multiplied with the specific gravity to convert it to mg/ml.

2.7. Chromatographic procedure

Exactly 20 μl of the sample solution and the standard solution were injected separately by means of the sample loop and chromatographed under the operating conditions described above. Quantitation was based on comparing the piperazine/internal standard peak area ratio of the sample to that of the standard.

2.8. Stability tests

About 800 mg of piperazine dihydrochloride were transferred into a 10-ml volumetric flask, dissolved, and diluted to volume with water. Three 0.5-ml aliquots of the solutions were each pipetted into separate 10-ml test tubes. After addition of 1 ml of one of the testing reagent described below, the tubes were tightly closed with PTFE-lined screw caps. The three tubes were heated at 80°C for 15, 30 and 45 min, respectively. At the end of each heating period, the tubes were immersed in an ice bath to cool the contents. The contents were extracted first with diethyl ether and then with chloroform. The remaining aqueous phase was adjusted to pH 14 with 8 *M* sodium hydroxide for test solutions treated with acid (1 *M* for solutions treated with hydrogen peroxide) or to pH 1 with 1 *M* hydrochloric acid for test solution with base, and again extracted with ether and chloroform as described above. Both extracts were evaporated to dryness under a gentle stream of nitrogen gas. The residues were reconstituted with 1.0 ml KH_2PO_4 buffer (pH 3.0), and chromatographed. The resulting chromatograms were evaluated by comparison with those obtained from control.

The following test reagents were used: *acid*: concentrated sulfuric acid; *base*: 1 *M* sodium hydroxide; *hydrogen peroxide*: 33.3% hydrogen peroxide; *control*: a 0.5-ml aliquot of the stock solution was first made alkaline with 1 *M* NaOH to pH 14, and extracted with ether and chloroform as above. The remaining aqueous phase was then acidified with 1 *M* hydrochloride acid to pH 1, and extracted also with ether and chloroform. Both extracts were subjected to the same treatment as the extracts with the test reagents.

In addition, three 0.5-ml aliquots, each in 10-ml tubes with PTFE-lined screw caps were exposed to short-wavelength UV light (254 nm) for 15, 30 and 45 min, respectively. Following acidification to pH 1 with 1 *M* hydrochloric acid, the solution was extracted with ether and chloroform as above. The aqueous phase was basified with 1 *M* sodium hydroxide to pH 14 and extracted also with ether and chloroform. The organic extracts were treated in a similar manner as described above.

2.9. Ruggedness test

The mobile phase factors: pH, potassium dihydrogenphosphate concentration, triethylamine content and flow-rate, along with column

temperature, water source and integrator attenuation were selected as the seven variables for Youden and Steiner's [22] ruggedness test. Each variable was studied at two levels, indicated by upper case and lower case letter in Table 1, to bracket the standard condition of the variable described under HPLC conditions. Two different batches of spiked tablet placebos were prepared, giving 1.42 and 2.84 mg/ml of solutions before chromatography, which were assayed following Youden and Steiner's experimental design shown in Table 1. The two levels of each variable studied are shown in Table 2.

2.10. Limit of quantitation

Exactly 1.0, 2.0 and 3.0 ml of a 1 mg/ml solution of piperazine dihydrochloride monohydrate solution were pipetted into three separate 10-ml volumetric flasks, each containing 65 mg of tablet placebos. After 1 ml of a 7 mg/ml solution of internal standard solution was added to each flask, the contents of each flask were diluted to volume with 0.07 *M* KH_2PO_4 buffer (pH 3.0), sonicated for 15 s, and filtered, discarding the first 5 ml of filtrate. Each filtrate was chromatographed seven times under the HPLC conditions described above and the peak area ratios calculated. The standard deviation of each set was calculated and plotted against the corresponding concentration level expressed in terms of amount of piperazine dihydrochloride monohydrate injected. The *y*-intercept of the regression line was calculated and multiplied by 10.

3. Results and discussion

Initial attempts to chromatograph piperazine with aqueous potassium diphosphate mobile phase (pH 3.0) resulted in broad tailing peaks. Addition of triethylamine to the mobile phase greatly reduced the tailing as triethylamine masked the polar silanol sites on the column particles. However, the amount of triethylamine in the mobile phase is critical; high concentrations of triethylamine resulted in poor resolution of piperazine from both solvent peak and internal standard peak.

Under the proposed experimental conditions piperazine and 1-phenylpropanolamine eluted as fairly symmetrical peaks with a tailing factor ($\pm$ S.D., $n = 20$) at 5% height of 1.38 ± 0.18 and 1.80 ± 0.01, respectively, and were well separated from each other with a resolution of 2.06 ± 0.11. The average retention times ($\pm$ S.D., $n = 20$), determined over a period of one month with five separately prepared mobile phases, were 4.93 ± 0.05 min for piperazine and 6.41 ± 0.04 min for 1-phenylpropanolamine with height equivalent to a theoretical plate (HETP) values

Table 1
Experimental design for Youden and Steiner's ruggedness test

Variable	Experiment No.							
	1	2	3	4	5	6	7	8
KH_2PO_4 Concentration[a]	A	A	A	A	a	a	a	a
pH	B	B	b	b	B	B	b	b
Triethylamine ratio	C	c	C	c	C	c	C	c
Flow-rate	D	D	d	d	d	d	D	D
Column temperature	E	e	E	e	e	E	e	E
Water source	F	f	f	F	F	f	f	F
Integrator attenuation	G	g	g	G	g	G	G	g
Observed results	s	t	u	v	w	x	y	z

[a] Upper case letter denotes high and lower case letter denotes low level of variable.

Table 2
Results of ruggedness test

Variables	Levels	Piperazine recovery (%)	
		1.42 mg/ml	2.84 mg/ml
KH_2PO_4 Concentration	0.075 *M* (A)	100.5	101.3
	0.065 *M* (a)	100.3	100.9
pH	3.10 (B)	99.5	100.0
	2.90 (b)	100.6	101.2
Triethylamine ratio	0.011 (C)	101.1	100.7
	0.009 (c)	100.3	99.9
Flow-rate	1.2 ml/min (D)	100.4	100.7
	0.8 ml/min (d)	100.4	100.3
Column temperature	26°C (E)	100.7	100.7
	22°C (e)	100.1	100.4
Water source	Milli-Q (F)	99.8	100.2
	Distilled (f)	101.2	100.5
Integrator attenuation	8 × (G)	100.5	99.8
	16 × (g)	100.3	101.2

(± S.D., $n = 20$) of 0.39 ± 0.07 mm and 0.36 ± 0.04 mm, respectively. Results showed that the piperazine peak appeared with the same retention time regardless whether it was injected as the free base, dihydrochloride, sulfate or citrate salt because of the low pH of the mobile phase. Piperazine with pK_{a1} of 9.83 and pK_{a2} of 5.56 (23.5°C) exists predominantly as the doubly protonated form at the mobile phase pH of 3.0 regardless of the initial salt form.

The relationship between piperazine/internal standard peak area ratio and the amount of piperazine injected was established. Linearity was obtained between 4 and 477 μg of piperazine dihydrochloride monohydrate injected ($r = 0.9994$). A typical regression equation for the standard curve was $A = 0.039C - 0.001$, where A = peak area ratio of piperazine/internal standard and C = amount of piperazine dihydrochloride monohydrate injected (μg). A similar linearity range was obtained upon injecting piperazine dihydrochloride or piperazine citrate.

In all recovery studies the dihydrochloride salt was used for the preparation of the standard solution since it was obtained with a higher degree of purity and it is easier to handle than the free base which is hygroscopic. Where appropriate, the equivalent amount of the piperazine dihydrochloride monohydrate of the salts was calculated for recovery study computations. The latter makes it possible for assaying the syrup because the commercial syrup contains piperazine sulfate. Attempts to obtain a pure sample of piperazine sulfate were unsuccessful. However, as is the case with the dihydrochloride and citrate salts, upon chromatographing piperazine sulfate, the piperazine peak appeared at the same location on the liquid chromatogram. The recovery studies were designed to investigate the recoveries of piperazine dihydrochloride by the proposed method from both spiked simulated tablet and syrup placebos. The placebos were prepared based on all possible excipients given for piperazine formulations [23] to cover as many different formulations

from different manufacturers. One batch of piperazine tablets was blue-colored. Qualitative analysis indicated that the coloring agent is FD&C No. 1. The studies covered a range from −33% to +33% of the label amount of piperazine or equivalent piperazine salts in commercial products. The chromatograms from these samples were similar to those of standard solutions. The tablet placebo gave a small extra peak before the piperazine peak at about 4 min, but did not interfere with it. No extraneous peaks were given by the syrup placebo between 1 and 10 min after injection. The overall percent recoveries (± S.D., $n = 6$) were 100.2 ± 0.8% for the spiked tablet placebo and 100.3 ± 1.0% for the syrup placebo spiked with piperazine dihydrochloride.

A ruggedness test was performed on the proposed method based on Youden and Steiner's experimental design [22] to determine if a small variation of an operating variable can be tolerated (Table 1). For example, to determine the effect of the KH_2PO_4 concentration on the assay result, the result averages of runs 1–4 were compared to those of runs 5–8. In other words, (s + t + u + v)/4 is compared to (w + x + y + z)/4 (Table 1). The experimental design shows that the other six factors appear twice at the high level and twice at the lower level in each set. Consequently, their effects on the results in each set are identical. Any difference in the results between the two sets must be due to the effect of KH_2PO_4. Results of this ruggedness test are shown in Table 2. In the case of the column temperature, the 26°C was achieved by using a column heating mantle. The standard deviations in percent recovery for both sample sizes are small indicating that this method is rugged within minor fluctuations of the seven operating variables.

The limit of quantitation was determined by the extrapolation method [24]. The y-intercept of the plot of standard deviation vs. the corresponding concentration represents the standard deviation of the analytical blank $S.D._0$. Multiplying $S.D._0$ by 10 provides an estimate for the limit of quantitation in terms of peak area [25]. Further comparison of this value with the peak area of a standard solution gives the limit of quantitation in terms of concentration. As measured the limit of quantitation was 5.3 μg of piperazine dihydrochloride monohydrate injected under the described experimental conditions.

The precision was determined by injecting the same sample seven times. The experiments were repeated three more times at 5-day intervals, using freshly prepared samples each time, and measuring the peak area ratios each time. The percent relative standard deviations were 1.75, 1.40, 1.63 and 1.13%, respectively.

The method was applied to the assay of commercial tablets and syrups. Fig. 1A shows a typical liquid chromatogram from commercial tablets. As with spiked tablet placebos, the

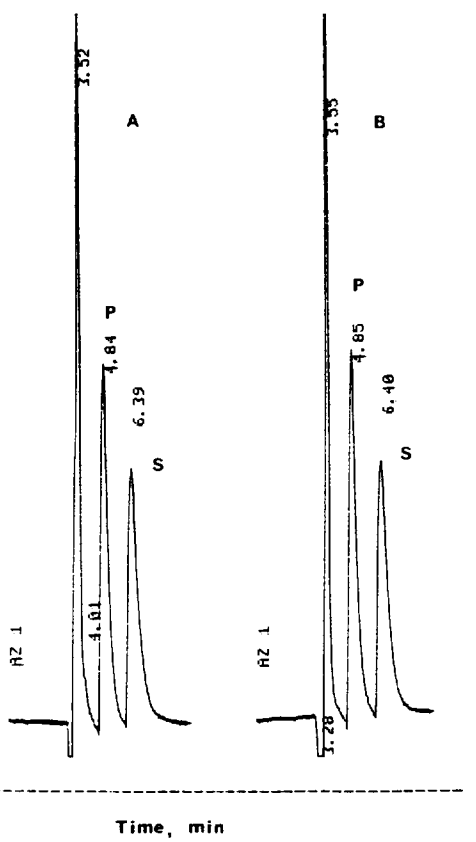

Fig. 1. Typical liquid chromatogram of an aqueous extract of a commercial tablet (A) and of a diluted solution of a commercial syrup (B). P = Piperazine; S = internal standard.

liquid chromatogram also showed an extra peak eluting before the piperazine peak at about 4 min. However, this extra peak co- elutes with the solvent peak and did not interfere with the piperazine peak. Fig. 1B is a liquid chromatogram from a commercial syrup. The assay results are shown in Table 3 which also includes the results obtained by the USP method for comparison. The results indicate that, in general, the compendial results gave consistently low results and may very well lead to a negative systematic bias. The compendial method is a gravimetric assay of piperazine after precipitating it as the picrate salt. The low results were presumably due to incomplete precipitation and/or losses during the washing of the precipitate with "absolute" ethanol. In spite of the discrepancies between the proposed and the compendial methods, a correlation ($r = 0.999$) is obtained between the two methods. The regression equation for the correlation line is USP = 0.989 · LC − 0.389 (USP = USP method; LC = HPLC method).

Semi-quantitative stability tests results showed that aqueous piperazine dihydrochloride solutions were relatively stable when subjected to acid or base at 80°C for 45 min because, within experimental errors, the area of the piperazine peak in the liquid chromatogram was similar in size as that of control. Subjecting the solution to short-wavelength UV light (254 nm) for 45 min did not change the size of the piperazine peak in the liquid chromatogram either. As expected, the compound was not stable toward hydrogen peroxide. The liquid chromatograms after the hydrogen peroxide treatment gave an additional peak, presumably of the mono- and/or dinitroso derivatives (Fig. 2). Fig. 2A is the liquid chromatogram of the acidic extract showing the extraneous peak X at about 5.4 min. Fig. 2B is the liquid chromatogram of the subsequent basic extraction to which internal standard was added. No effort was undertaken to identify the extraneous peaks. The oxidation products eluted at different retention times which means that the oxidized products can be detected by the proposed assay method, and they did not interfere

Table 3
Assay results of commercial formulations by the proposed and USP methods

Label claim (mg/tablet or ml)	Label claim (%)[a]	
	HPLC	USP
Tablet		
50	99.0	92.4
50	98.4	92.2
250	98.1	93.6
250	97.9	95.1
Syrup		
340	99.8	96.5
340	101.2	101.2

[a] Average of duplicate results.

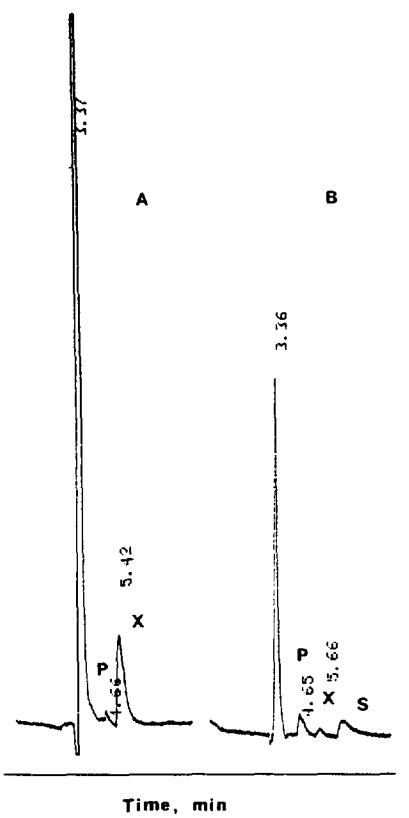

Fig. 2. Liquid chromatogram of piperazine after H_2O_2 treatment at 80°C for 30 min. (A) Acidic extract; (B) subsequent basic extract spiked with internal standard (S). P = Piperazine; X = extraneous peak.

with the piperazine peak. Samples in strong acid, in strong base, and in strong hydrogen peroxide required extensive dilution before injecting on the column. The low amounts of piperazine used in the studies will become so diluted that degradation compounds may not be detected, if the diluted solutions were directly injected onto the column. Consequently, the samples were simply extracted under acidic as well as basic conditions as described. Since the control needed to be extracted also, the samples which were subjected to UV radiation, were also extracted.

The above results indicate that the method is simple and provides quantitative, reproducible results for the assay of piperazine in commercial formulations for quality control purposes. The method is rugged for the seven variables tested. For the dosage forms, the proposed method is safer and requires a much shorter assay time than the USP gravimetric method.

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