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Determination of hydrazine in hydralazine by capillary gas chromatography with nitrogen-selective detection after benzaldehyde derivatization[a]

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ABSTRACT

A method for the determination of hydralazine substance is described. Hydrazine is derivatized in aqueous media with benzaldehyde to benzalazine. After extraction to an organic phase containing a homologue as marker, the sample is subjected to capillary column gas chromatography with nitrogen-selective detection. A prolonged reaction with 0.1 *M* benzaldehyde of 20 min or more led to an increased level of benzalazine when hydralazine was analysed. An increase was also observed if the aqueous hydralazine sample had been allowed to stand for some time before analysis. The final method involved the use of a 5-min reaction time, fresh solutions and the standard addition principle. The levels of hydrazine found in hydralazine hydrochloride were below 1 ppm (as bases, 1 ng/mg).

INTRODUCTION

Hydralazine (Fig. 1) is a cardiovascularly active compound that has been used as an antihypertensive agent since the 1950s. In combination with β-adrenoreceptor blocking agents the daily dose can be reduced, thus minimizing unwanted side-effects.

Attention has been paid to the presence of hydrazine (Fig. 1) in different drugs

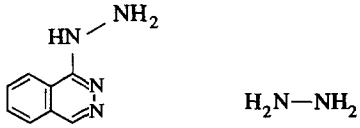

Fig. 1. Hydralazine (left) and hydrazine.

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after reports about its mutagenic effects in laboratory animals[1]. Contamination of hydralazine with hydrazine may occur during synthesis or by degradation during storage of the substance or the pharmaceutical formulation.

As hydrazine is a small and polar molecule, most methods for the determination of low concentrations are based on derivatization followed by chromatographic separation of the derivative. Several papers on the determination of hydrazine in different pharmaceutical formulations containing hydrazinic ingredients have been published, including isoniazid[2], phenelzine[3], hydralazine and isoniazid[4] and isocarboxazid[5]. Most of these methods are based on the reaction with an aromatic aldehyde[2–4] (Fig. 2). Attempts to determine hydrazine in hydralazine by liquid chromatography led to irreproducible results, and a gas chromatographic method was therefore developed[4]. The problem with liquid chromatography is interference from the excess of Schiff base formed from the original hydrazinic drug substance itself, which only eluted after several injections as an increasing baseline[4]. This problem can be eliminated by the the use of a short packed disposable column prior to the chromatographic analysis[6].

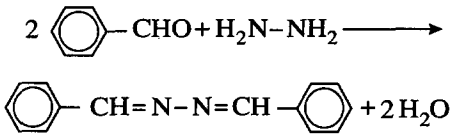

Fig. 2. Reaction of benzaldehyde with hydrazine to give benzalazine.

Gas chromatography for the simultaneous determination of hydrazine and benzylhydrazine in isocarboxamide was at first impossible, as the hydrazone formed was not stable[5], but by derivatization to a pyrazole derivative using a diketone as reagent a stable derivative was obtained[5]. 2-Hydroxy-1-naphthaldehyde has also been used for derivatization followed by UV[7] and fluorimetric[8] detection. Recently a method for the determination of hydrazine and acetylhydrazine in blood after derivatization with pentafluorobenzaldehyde was reported[9].

Some interest in hydrazine determinations has been focused on boiler feedwater, where it is used as an antioxidant. Proposed methods involve the use of electrochemical detection after liquid chromatography of free hydrazine[10] or after salicylaldehyde derivatization[11].

To develop a method for the determination of hyrazine in hydralazine we adopted the gas chromatographic method described by Lovering and co-workers[2–5] (Fig. 3a) with some modifications. These include the use of a capillary column instead of a packed column and a homologue of benzalazine as a marker in the organic phase instead of 5-chloromethyl-2-aminobenzophenone[4].

EXPERIMENTAL

Apparatus

Gas chromatograph. A Varian 3700 gas chromatograph was equipped with a nitrogen–phosphorus-selective detector and a capillary column. To fit the column, the instrument was modified with a Gerstel adaptor at the detector side and a Ger-

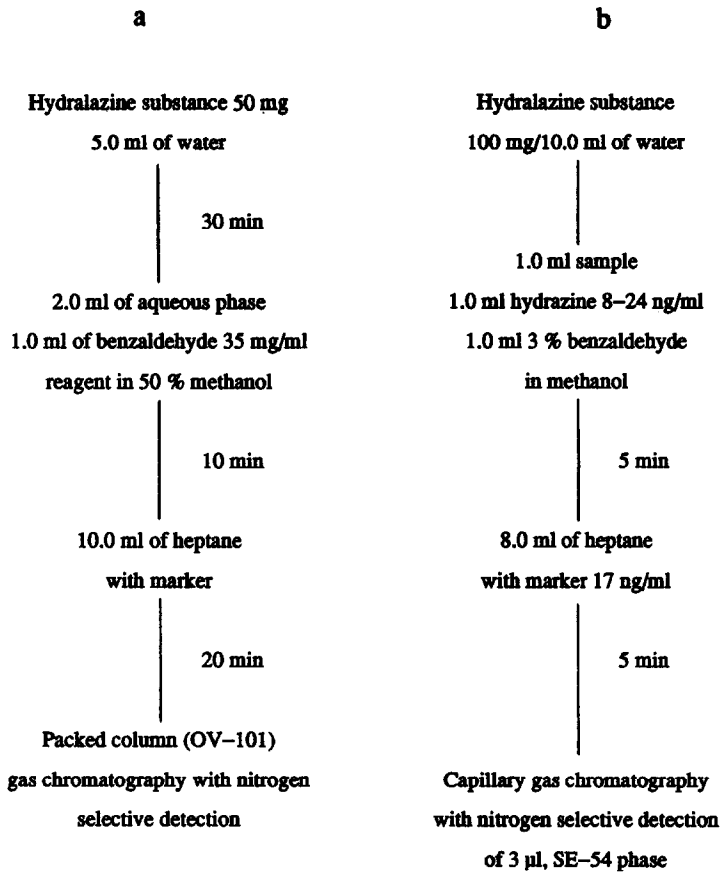

Fig. 3. Schemes for the analysis of hydrazine by gas chromatography with nitrogen-selective detection after derivatization. (a) Previous method[4]; (b) present method.

stel–Swagelock adaptor at the injector side. The fused-silica column (25 m × 0.32 mm I.D.) was coated with SE-54 (0.25 μm). During use *ca.* 20 cm was cut off when required to restore the peak symmetry. Injections were made with the split valve closed. After 2 min it was opened automatically, controlled by a separate unit. It was closed prior to the next injection by a signal from the auxiliary channel of the auto-sampler.

The detector bead voltage was 4 V and the current setting was between 300 and 900 depending on the actual bead probe used and its age. The input current to the detector was adjusted so that a background current of *ca.* 10 mV was obtained.

The instrument temperatures were injector 280°C, detector 300°C and oven 100°C (1 min), increased at 15°C/min to 285°C, which was maintained for 1 or 5 min depending on the type of sample.

The inlet pressure of the nitrogen carrier gas was 100 kPa. Gas flow-rates to the detector were make-up nitrogen 20 ml/min, air 175 ml/min and hydrogen 4.5 ml/min. With a recent detector probe the hydrogen flow-rate was increased to 5.5 ml/min in

order keep the bead working after the solvent had passed, otherwise the "flame" was extinguished and sometimes failed to re-ignite.

Injections. A Varian 8000 autoinjector was used. The auxiliary time facility of the control unit was used to close the split before the next injection. A 3-μl volume was injected throughout. Occasionally the whole injector system was flushed with methanol to prevent precipitation in the tubing by the hydralazine hydrazone from the non-polar heptane phase when the system was at rest. At the same time the syringe piston inlet was cleaned with the same solvent.

Integrator. Chromatograms were recorded and evaluated with a Hewlett-Packard 3390A integrator. Normally peak areas were reported but peak heights gave slightly better precision at low concentrations levels.

The samples were shaken mechanically in an upright position without caps (Ika-Vibrax-VXR; Janke & Kunkel, Staufen, F.R.G.). Up to 36 tubes of 15 ml can be handled simultaneously with this apparatus.

Reagents and chemicals

Hydrazine monohydrochloride (purum; Fluka, Buchs, Switzerland), benzaldehyde (puriss; BDH), 3- and 4-fluorobenzaldehyde (purum; Fluka), benzalazine (Koch-Light Labs., Colnbrook, U.K.), phenylhydrazine, 4-methylbenzaldehyde (*p*-tolualdehyde) (purum, Fluka) and hydralazine hydrochloride (Ciba-Geigy, Basle, Switzerland, batch 37-187-16, s 40 843-2, 870422). Solvents and the buffer salts used were of analytical-reagent grade.

4-Fluorobenzalazine used as a marker was prepared in crystalline form as follows: 4.28 mg of hydrazine hydrochloride was dissolved in 5.0 ml of water + 5.0 ml of methanol, then 50 μl of 4-fluorobenzaldehyde were added and after about 5 min a precipitate formed. After centrifugation the supernatant was decanted and discarded. The yellow crystals were washed twice with 10% aqueous methanol and dissolved in dichloromethane. The solution was placed in a small glass vial, the solvent was evaporated with nitrogen and the crystals obtained were stored in the vial.

Benzaldehyde reagent was prepared by diluting 3.0 ml of the aldehyde to 100.0 ml with methanol. This solution was not kept for more than 1 week.

Determination of hydrazine

Preparation of the samples. Deionized water was used for the preparation of all hydrazine and hydralazine samples. A 63-mg amount of hydralazine hydrochloride was dissolved in 5.0 ml of water (equivalent to 10 mg/ml of hydralazine base). To 2.0 ml of the solution was added 1.0 ml of a 3.0% solution of benzaldehyde in methanol. An Eppendorf repeater pipette was used for addition of the reagent. The mixture was shaken in an upright position for 5 min (before it was found that additional benzalazine was formed in the aqueous phase after about 10 min owing to degradation of the hydralazine Schiff base, the derivatization time adopted at first was 20 min). The reaction mixture was then extracted with 8.0 ml of heptane containing the marker 4-fluorobenzalazine (17 ng/ml). The heptane was dispensed with a Socorex (Renens, Switzerland) 511-10 dispenser.

Both reaction and extraction were performed in 15-ml culture tubes with mechanical shaking. After extraction for 5 min, about 1 ml of the organic upper phase was decanted into a glassvial for autoinjection into the gas chromatograph. A 3.0-μl

volume was injected twice if not stated otherwise. The ratio of peak area or height of benzalazine to that of the marker was calculated and plotted against concentration.

Standard solutions. A hydrazine stock solution (20 μg/ml) was prepared by dissolving 4.28 mg of hydrazine hydrochloride in 100.0 ml of water. The solution was then further diluted to obtain three standard solutions of 8, 16 and 24 ng/ml. These standards were prepared from a fresh stock solution for each analysis. They were used within 30 min and then discarded.

Calibration graphs. For the preparation of the calibration graphs 2.0 ml of each standard solution were analysed as above (duplicates). The derivatization time was 20 min.

Standard addition procedure. For the standard addition procedure the concentration of the hydralazine solution was 20 mg/ml and 1.0 ml of this solution was mixed with 1.0 ml of hydrazine hydrochloride standard solution (0, 8, 16 and 24 ng/ml, as hydrazine). The analysis was performed as described above. The derivatization time was 20 min for the first analyses, and 5 min for the latter ones, for the reason explained below.

RESULTS AND DISCUSSION

Capillary gas chromatography of azines

Capillary columns with neutral or slightly basic characteristics were chosen for the gas chromatography of benzalazine. Symmetrical peaks were observed and normally no carryover of samples in the system was noted. Occasionally, after several injections, the peaks on the SE-54 column showed a tendency to tail. This was eliminated by cutting off the first 20 cm of the column when necessary.

Choice of marker

One intention of this study was to find a more suitable marker for the chromatographic system. Matsui *et al.*[4] used 5-chloro-2-methylaminobenzophenone as an internal standard with a packed column. Preliminary experiments were performed with the azine of tolualdehyde as marker. A drawback with this marker was its difference in retention time compared with benzalazine, *i.e.*, about a 4 min longer time at the rate of 6°C/min and 60 kPa hydrogen inlet pressure. In the present method, with a rate of temperature increase of 15°C/min, the corresponding retention time difference was less than 2 min, which still is considered to be too long. The precision on repeated injections of a solution of the derivatives was 6.5%. In order to improve the accuracy of the chromatographic step a marker with a retention time closer to that of the derivative was desired.

It was found that 3- and 4-fluorobenzalazine both eluted 0.3 min earlier than benzalazine and were stable in heptane for several days. 4-Fluorobenzalazine was chosen as a marker owing to the good precision of the peak response ratios of benzalazine to marker. The relative standard deviation (R.S.D., n = 7–9) was 0.7% compared with 1.5% for the 3-fluoro derivative. This is better than the precision obtained using the azine with tolualdehyde as marker (Table I).

The other two markers tested were based on phenylhydrazine. The benzaldehyde derivative formed had a 0.3 min longer retention time than benzalazine and the R.S.D. was 16% (n = 5). The stability in excess benzaldehyde was poor (about 80%

TABLE I

RETENTION TIMES OF BENZALAZINE AND POSSIBLE MARKERS ON THE SE-54 CAPILLARY COLUMN

Column, 25 m × 0.32 mm I.D. with 0.25-μm SE-54; carrier gas, 100 kPa nitrogen inlet pressure; oven temperature, 100°C for 1 min then increased at 15°C/min; *ca.* 60 pg of each as hydrazine in heptane injected.

Retention time (min)	*Calculated elution temperature, (°C)*	*Compound*	*R.S.D.[a] (n = 7–9) (%)*
8.58	214	3-Fluorobenzalazine	1.5
8.61		4-Fluorobenzalazine	0.7
8.90	219	Benzalazine	
9.36		Phenylhydrazine phenylhydrazone	16
10.0	235	Phenylhydrazine tolylhydrazone	–[b]
10.6	244	Tolualdehyde azine	6.5

[a] Based on the peak-area ratio of benzalazine to the actual compound.
[b] Not measured.

loss after 24 h). The poor stability of the hydrazones of phenylalkylhydrazines with benzaldehyde has also been reported by Lovering *et al.*[5]. The retention time for the tolualdehyde derivative of phenylhydrazine was 1.1 min longer than that for benzalazine. The retention times of benzalazine and the different markers are summarized in Table I.

Reaction time

The optimum reaction time for hydrazine with 1% benzaldehyde in aqueous methanol was first investigated for 800 ng/ml aqueous hydrazine solutions. We used 33% methanol because at only 17%[4] droplets of benzaldehyde were present in the solution. The reaction was stopped by the addition of and extraction with heptane. It was found that an extracted and decanted sample was stable for several hours, even if the derivatization was not complete.

A plateau appeared after 15–20 min in the reaction time profile for a hydrazine solution of 800 ng/ml (Fig. 4). When hydralazine hydrochloride was included in the reaction mixture, 5 min seemed to be sufficient (Fig. 5). As no increase in benzalazine was noticed for longer reaction times (20–60 min) in any of these time-course profiles at the level of 800 ng/ml, 20 min was considered to be an adequate but not too long time for the reaction. In the original GC method[4] the derivatization time was 10 min followed by extraction for 30 min (Fig. 3a). Our own experiments showed that 2.5 min was sufficient for quantitative extraction and 5 min was selected for this step.

These conditions were then used for the analysis of aqueous standard solutions of hydrazine hydrochloride. Linear graphs were obtained with the intercept near the origin, *e.g.*, Fig. 6. The precision was acceptable (R.S.D. 8.4% at 20 ng/ml, $n = 9$). However, when standard addition analysis was performed on hydralazine hydrochloride the spread increased. The level of the samples without added hydrazine were often far from the curve. This prompted us to re-evaluate the reaction conditions in the actual low concentration range encountered. When hydrazine was added at 20 ng/ml, a significant increase in the benzalazine formed after a reaction time of about

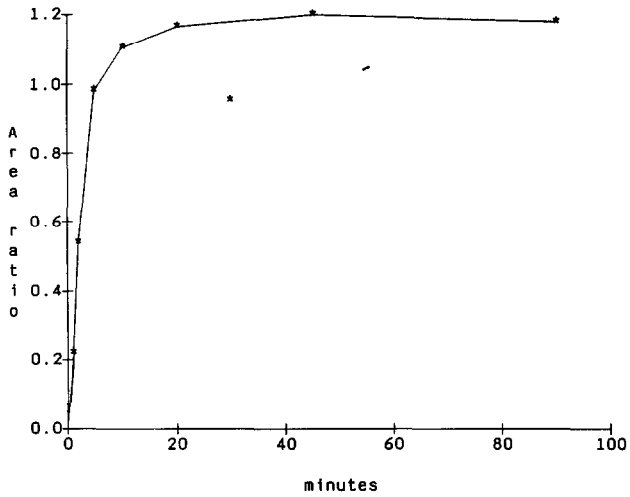

Fig. 4. Reaction time profile: formation of benzalazine from hydrazine hydrochloride, 800 ng/ml as base, with 1% benzaldehyde (0.098 *M*).

10 min was observed (Fig. 7). This increase can be explained either by degradation of the hydralazine Schiff base to hydrazine followed by condensation with benzaldehyde, or by a direct reaction of benzaldehyde with the Schiff base. A similar pathway has been proposed for the reaction of the acetone hydrazone of hydralazine with pyruvic acid[12]. The high hydrazine concentration used earlier of 800 ng/ml might explain why an increase in benzalazine was not observed for longer reaction times in the first rection time profiles. A plateau appeared between 2.5 and 7.5 min in most profiles at 20 ng/ml, and then benzalazine increased irregularly with time. The duration of the plateau was shortened when the samples were not shaken during the

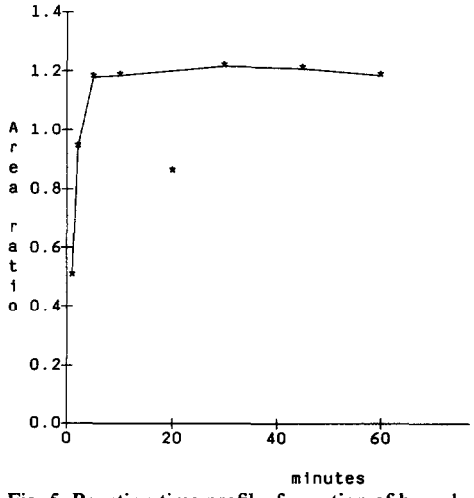

Fig. 5. Reaction time profile: formation of benzalazine from hydrazine hydrochloride, 800 ng/ml as base, with 1% benzaldehyde (0.98 *M*) in the presence of hydralazine hydrochloride, 20 mg/ml (as base).

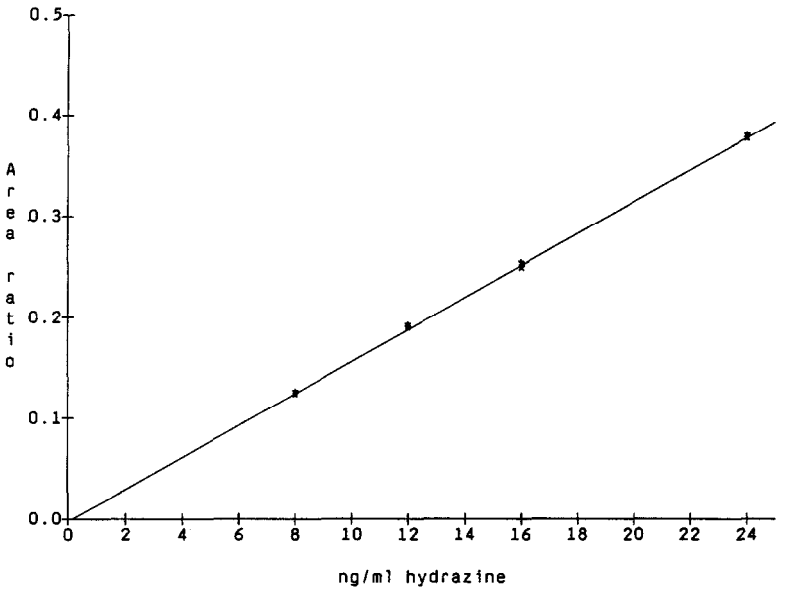

Fig. 6. Calibration graph for the determination of aqueous hydrazine hydrochloride. Marker concentration: 17 ng/ml in heptane (as hydrazine). Each point on the curve is the mean of two injections. Regression equation: $y = 0.016x + 0.0036$ ($r = 0.99993$).

reaction. The reason for this could be that the precipitation of the hydralazine Schiff base was now slower in the reaction tubes. Dissolved Schiff base can be expected to decompose at a faster rate when in solution than if it is present as crystals.

These results prompted a reduction in the derivatization time to 5 min, which was used for all further determinations. It can be concluded that if the actual reaction

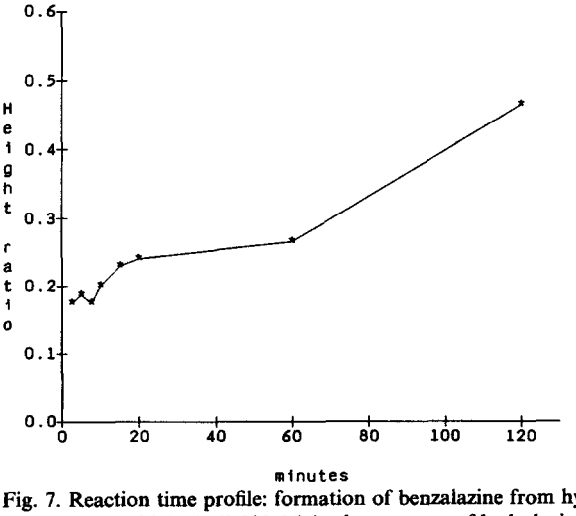

Fig. 7. Reaction time profile: formation of benzalazine from hydrazine hydrochloride, 20 ng/ml as base, with 1% benzaldehyde (0.098 *M*) in the presence of hydralazine hydrochloride, 20 mg/ml (as base).

time is too short this will only affect the slope of the standard addition curve and not the intercept on the abscissa. On the other hand, if the reaction time is too long benzalazine will be formed in equal amounts in all samples from hydralazine and the curve thus moves up parallel to the theoretical curve and the intercept on the abscissa thus increases. This will lead to falsely high levels of hydrazine. The final method is outlined in Fig. 3b.

Stability of solutions and derivatives formed

Hydrazine solutions. A solution containing 20 ng/ml decomposed by 6% during 2 h and by 50% during 24 h, as can be seen in Fig. 8. Even the hydrazine stock solution (20 μg/ml) was found to be unstable. A loss of about 46% was observed after 2 weeks at room temperature in the dark.

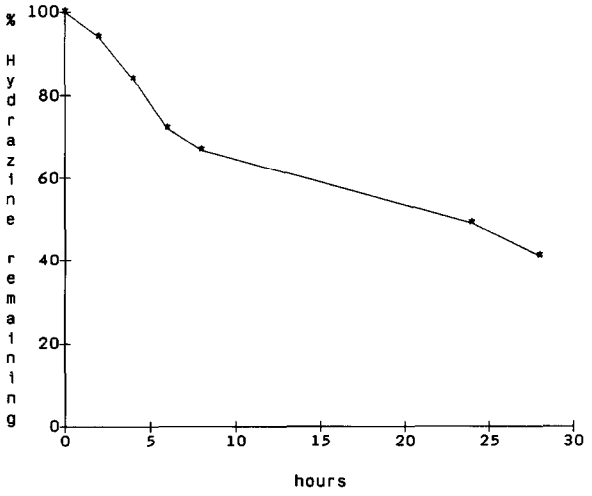

Fig. 8. Time course for the decomposition of a diluted hydrazine solution given as a percentage of the orginal concentration. A stock solution of hydrazine hydrochloride was diluted to 20 ng/ml (as base), from which 2.0-ml duplicates were withdrawn and analysed after reaction with benzaldehyde for 20 min.

Matsui *et al.*[2] reported that the stability of the dihydrochloride was superior to that of the sulphate or hydrate for their calibration standard of concentration 24 μg/ml. The stock standard solution was used during the day of its preparation and the working standards prepared by dilution were used immediately.

Hydralazine solution. A 10 mg/ml aqueous hydralazine solution was shown to decompose. After 1 h an increase in hydrazine as benzalazine of 21% was observed, and after 5 hours it was *ca.* 200%. The time course is illustrated in Fig. 9. This instability has been mentioned elsewhere[2].

Benzalazine. Butterfield *et al.*[3] reported that benzalazine is not stable in the pH 3.5 aqueous reaction medium used when hydrazine sulphate was the starting material. Significant decomposition started after 20 min with benzaldehyde in unbuffered media. With hydrazine dihydrochloride as the starting material the benzalazine formed was stable for at least 35 min[2].

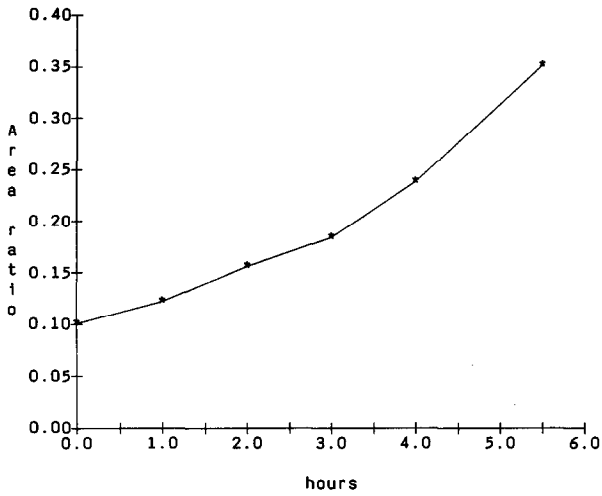

Fig. 9. Time course for the formation of hydrazine in an aqueous hydralazine hydrochloride solution, 10 mg/ml (as base). Duplicates of 2.0 ml were withdrawn and analysed by reaction with benzaldehyde for 20 min followed by extraction.

When investigating liquid chromatography with mass spectrometry (LC–MS) as a possible technique for the determination of hydrazine in hydralazine, the reaction with benzaldehyde was performed with a dodecadeuterated benzalazine marker present. This deuterated compound was partly converted to benzalazine[13], indicating that transfer or exchange of the aldehyde moiety can occur. This observation was investigated further and confirmed by an experiment in which the transformation of

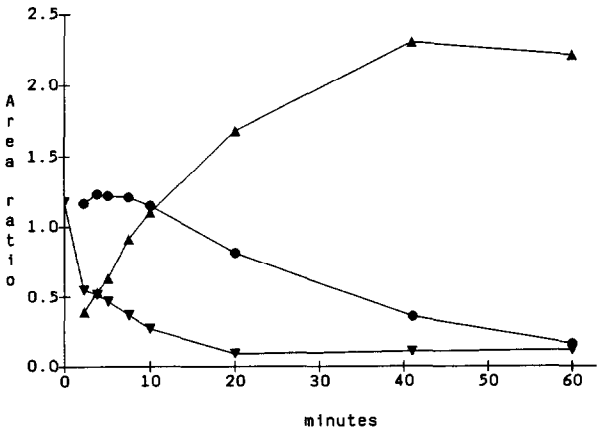

Fig. 10. Time course for the transformation of benzalazine to an azine with tolualdehyde (0.011 *M*). Benzalazine (60 μg) was dissolved in 3.0 ml of methanol and 6.0 ml of buffer (pH 3.0) (ionic strength = 0.05, phosphate). Just before time zero, one 0.5-ml sample was withdrawn and extracted with 1.5 ml of heptane containing 100 ng/ml of the marker. At time zero, 10 μl of tolualdehyde were added, the tube was kept agitated and samples were withdrawn at suitable time intervals for analysis as described. ▼, Benzalazine; ●, mixed azine derivative; ▲, azine with tolualdehyde.

benzalazine to an azine with tolualdehyde was studied. The time course is shown in Fig. 10. The actual concentration of the aldehyde was reduced to 0.1% as with the standard 1% concentration of reagent the exchange was virtually instantaneous. Even with this lower concentration only 50% of the initial benzalazine concentration remained after 2 min. Simultaneously the mixed azine has its maximum at about 4 min Fig. 10). Similar transformation has been reported when benzalazine was mixed with pentafluorobenzaldehyde at pH 5.2[14] using a molar ratio of 1:20. Here the reaction was slower, 50% of benzalazine remaining after 2 h.

Hydralazine Schiff base. Formation of additional benzalazine from the hydralazine Schiff base in the heptane phase (after extraction) seemed to be serious in the presence of the aqueous phase. The initial benzalazine content was doubled after 5 h. However, once the separated heptane phase was decanted into a glass vial, the derivative was found to be stable for several days. Repeated analyses of decanted samples gave a benzalazine-to-marker ratio within 5%. This indicates that the benzaldehyde reagent mainly remained in the aqueous phase and/or that a protic solvent is required for the transformation.

Reaction yield and precision of the method

The quantitative reaction yield for the derivatization of aqueous hydrazine solutions was about 86% for 12 ng/ml and 94% for 24 ng/ml. References were heptane solutions of pure benzalazine of about the same concentrations with respect to hydrazine. The precision of analysis was determined by analysing ten samples of derivatized and extracted hydrazine (20 ng/ml). The amount of benzalazine injected was 98 pg, and the R.S.D. of the area count ratio of benzalazine to that of the marker was 7.7% (6.5% for $n = 9$).

Analysis of hydralazine batches

Owing to the low levels of hydrazine found in preliminary determinations the standard addition principle was considered to give more accurate values than calculation from calibration graphs. The levels of hydrazine in ppm found in different hydralazine batches 1–2 years old are presented in Table II. An example of a standard addition curve is presented in Fig. 11 and a series of representative gas chromatograms are shown in Fig. 12. The levels found were in the range 0.3–0.4 ppm. These values are near the limit of quantification with the present method.

TABLE II

HYDRAZINE IN HYDRALAZINE SAMPLES

Analysis by standard addition of hydrazine and a reaction time of 5 min with benzaldehyde.

Sample code	*Source*	*Hydrazine (ppm)*
A = 064675 01	Ciba-Geigy	0.27
B = 063495 01	Ciba-Geigy	0.39
H = 072069 01	Ciba-Geigy	0.34
F	See *Reagents and chemicals* section	0.29

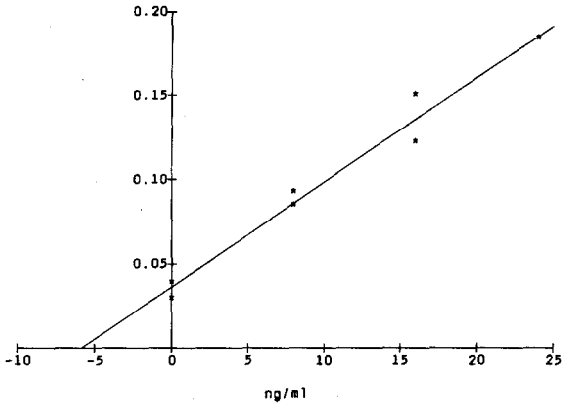

Fig. 11. Standard addition curve for hydrazine in hydralazine hydrochloride, batch F, 20 mg/ml (as base). Each point on the curve is the mean of two injections. Regression equation: $y = 0.0082x + 0.026$ ($r = 0.9234$). Concentration found: 5.8 ng/ml, which corresponds to 0.29 ppm.

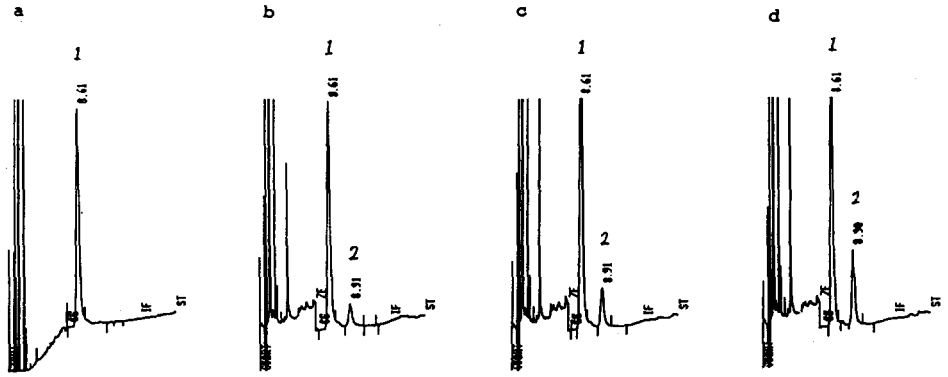

Fig. 12. Gas chromatograms with nitrogen-selective detection after analysis of hydralazine, 20 mg/ml. (a) Blank; (b) no hydrazine; (c) 8 ng/ml of hydrazine added; (d) 24 ng/ml of hydrazine added. Peaks: 1 = 4-fluorobenzalazine (marker), 17 ng/ml as hydrazine; 2 = benzalazine. Chart speed: 0.2 cm/min, and 2.0 cm/min from 8.5 min. SE.54 column. For other details, see Experimental.

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