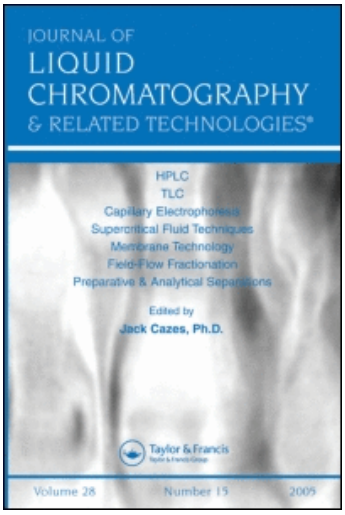

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ANALYTICAL METHOD FOR 1-METHYL-4-AMINO-PIPERAZINE IN AN ACTIVE PHARMACEUTICAL INGREDIENT USING CHEMICAL DERIVATIZATION AND HPLC-UV

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□ *A method to form a UV-active derivative of 1-methyl-4-amino-piperazine (AMP) was developed. The method was based on the reaction of AMP with benzaldehyde, forming a stable derivative, which was UV-active. The method was used to analyze samples of an active pharmaceutical ingredient (API) for trace amounts of AMP. The derivatization approach allowed detection of AMP at low levels, using readily available HPLC-UV instrumentation. The method was validated in the range of about 30 to 190 ppm (on a mass to mass basis relative to API). It was shown through spiked recovery tests that the derivatization reaction occurred smoothly without interference from the API. The results suggest that other organohydrazine compounds may be amenable to the same derivatization technique.*

Keywords benzaldehyde, benzylidene, derivatization, hydrazine, hydrazone, organohydrazine

INTRODUCTION

Detection of trace impurities in an active pharmaceutical ingredient (API) is a significant challenge in the pharmaceutical industry. Recently, the organic synthesis of an API under development at Novartis utilized 1-methyl-4-amino-piperazine (AMP) in the final step of the synthesis. Due to the potential toxicity of AMP, an analytical method was required so that low levels of AMP could be detected in the API, in order to provide rapid feedback to synthetic chemists who were experimentally comparing various purification schemes of the API. Under such

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circumstances, liquid chromatography with UV detection (HPLC-UV) would normally be the method of choice, due to the suitability for analysis of large numbers of samples. However, AMP itself is not UV active, meaning that direct detection of AMP by HPLC-UV was not a viable option. Therefore, a chemical derivatization reaction to render AMP UV active, followed by HPLC-UV analysis, was considered as an alternative.

Since AMP contains a hydrazine functional group, in principle it is suitable for conversion to a hydrazone derivative, based on the well known reaction of organo-hydrazine type compounds with aldehydes and ketones. Prior to modern spectroscopic techniques, this reaction was widely used for analysis of aldehydes and ketones. The aldehyde or ketone was typically reacted with 2,4-dinitrophenylhydrazine (DNP), and the acid or ketone was identified by measuring the melting point of the resulting crystalline hydrazone derivative.[1] More recently, quantitative analysis of aldehydes and ketones, either individually or in mixtures, has been accomplished by first reacting the carbonyl analytes with DNP or another suitable organo-hydrazine to produce the hydrazone derivative, and then analyzing the derivatives by liquid or gas chromatography.[2–5]

Although this reaction has been extensively used for analysis of aldehydes and ketones, it is also possible to utilize the reaction in a complimentary way, for analysis of hydrazine or organohydrazines such as AMP. Examples of the reaction being used in this manner have been reported,[6–9] but such reports are rare. Of particular interest to us was a report describing this approach for analysis of hydrazine in a pharmaceutical product.[10] It was shown that the reaction of hydrazine with two equivalents of benzaldehyde produced benzalazine, which was readily analyzed by GC, allowing one to determine the amount of hydrazine which was originally present in the pharmaceutical product. This finding suggested that benzaldehyde may also be a suitable reagent for organohydrazines, reacting in a 1:1 ratio to form a benzylidene type derivative. Such a derivative would presumably be UV active, making the approach suitable for HPLC-UV analysis of UV inactive organohydrazines such as AMP. We therefore investigated this approach for analysis of AMP in samples of the API under study.

EXPERIMENTAL

Chemicals

Chemicals were reagent grade or better. Acetonitrile (MeCN) and methanol (MeOH) were HPLC grade and water was purified using a Purelab Ultra system (Elga Labwater, Lowell, MA, USA).

HPLC-MS Instrument

Structural confirmation of the expected derivatization reaction product was obtained using an "open access" HPLC instrument with a mass spectrometry (MS) detector, shared by several research and development groups within Novartis Chemical and Analytical Development. The instrument consisted of Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA), equipped with a Micromass Quattro Micro API detector (Waters Corp., Milford, MA, USA) and a Symmetry C_{18} column of dimensions 3×150 mm, 3.5 um (Waters) maintained at 40 degrees. Flow rate was 0.5 mL/min. A gradient of 10 to 90% MeCN over 14 minutes was used, with 0.1% formic acid in the mobile phase.

HPLC-UV Instrument

The HPLC-UV system consisted of an Alliance 2695 separations module and 996 photodiode array UV detector (Waters). The column was Zorbax SB C_{18} (Agilent) of dimensions 150×3 mm, dp 3.5 µm, maintained at temperature of 40 degrees. Flow rate was 0.8 mL/min and injection volume was 20 µL. Mobile phase A was 0.1% TFA in water, mobile phase B was 0.1% TFA in MeCN. The gradient program was 2 to 35% B over first 4 min, hold at 35% B from 4 to 8 min, 35 to 100% B from 8 to 12 min. The detection wavelength of 290 nm was used.

Derivatization Procedure

API sample preparation: Dissolved 50 mg of API into 80 mL of MeOH in 100 mL volumetric flask. Added 1 mL of stock benzaldehyde solution (1% v/v in MeOH), then diluted to final volume of 100 mL. Allowed to react 24 h before HPLC analysis.

AMP reference standards: Prepared AMP solution of 0.5 µg/mL by serial dilution. Pipetted 3, 5, and 10 mL volumes of 0.5 µg/mL AMP solution into three 100 mL volumetric flasks. Added 0.5 mL of stock benzaldehyde solution (1% v/v) to each flask and diluted to final volume of 100 mL, to obtain three AMP-benzylidene derivative standards of 0.0015, 0.0025, and 0.005 µg/mL (equivalent to 30, 50, and 100 ppm AMP, relative to 0.5 mg/mL nominal API concentration). Allowed to react 24 h before HPLC analysis.

RESULTS AND DISCUSSION

Reaction of AMP with Benzaldehyde

Several preliminary experiments were run to evaluate the reaction of AMP with benzaldehyde as shown in Figure 1. In the first experiment,

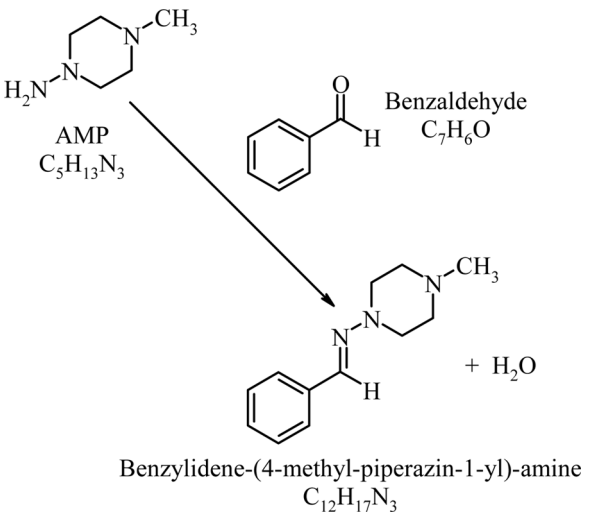

FIGURE 1 Condensation of amino-methyl-piperazine (AMP) with benzaldehyde to obtain benzylidene-(4-methylpiperazin-1-yl)-amine.

AMP was combined with 2.5 equivalents of benzaldehyde. Two more experiments were run in parallel in which 1 eq and 2.5 eq of acid (HCl) was also added, to see if the acidity of the solvent medium had any effect on the reaction. In all three cases, HPLC-UV showed the rapid appearance of a new peak, which was confirmed by HPLC-MS as having the molecular weight of the expected benzylidene derivative (ES^+, calculated exact mass 203.14, observed $[MH]^+ = 204$). These experiments indicated that the reaction was relatively insensitive towards variations in acidity of the medium, an important factor considering that the API was a malonate salt, meaning that one equivalent of malonic acid would be present in samples of API.

Additional experiments were conducted to quantitatively evaluate the extent of the derivatization reaction. In the case of the AMP reaction, monitoring the extent of the reaction was challenging. The major problem was that AMP itself was not detectable by UV, thus disappearance of AMP could not be measured directly by HPLC-UV. Another problem was the large difference in UV spectra of the benzaldehyde and the AMP-benzylidene derivative, as observed by photodiode array UV spectral analysis (Figure 2). The major absorbance peak in the UV spectrum of the AMP-benzylidene derivative was significantly red shifted relative to benzaldehyde, a general phenomenon for hydrazone derivatives of benzaldehyde and other aromatic aldehydes.[11] Lacking an isolated authentic reference standard of the AMP-benzylidene derivative, there was no rational way of selecting a wavelength which insured equal detector response between benzaldehyde

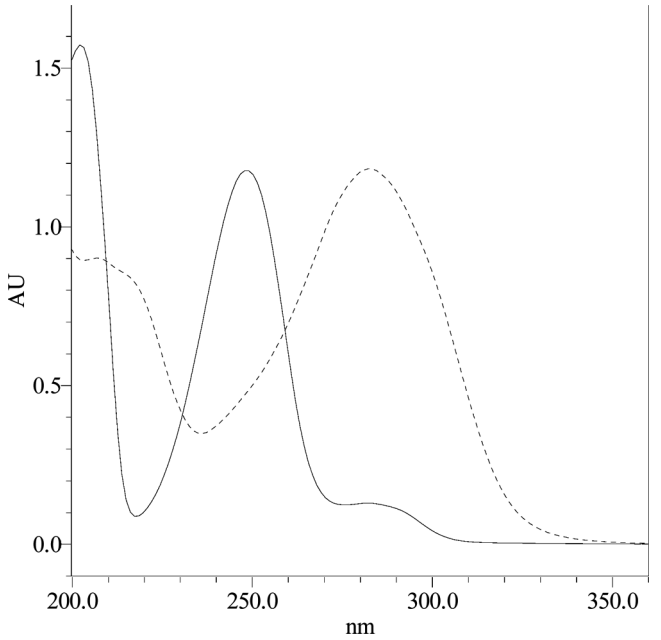

FIGURE 2 UV absorption spectra obtained with photodiode array HPLC detector. Solid line is for benzaldehyde, dashed line is for AMP-benzylidene derivative. Maximum absorption wavelength of benzylidene (282 nm) is red-shifted by 34 nm relative to benzaldehyde (248 nm).

and the AMP-benzylidene derivative. Therefore, another approach was used to establish the extent of reaction. This was accomplished by adding a measured excess of benzaldehyde relative to AMP, and monitoring the decrease in concentration of benzaldehyde by HPLC-UV. Two reactions were run with initial AMP concentrations of about 10 mM, using two different amounts (2.1 and 4.4 eq) of benzaldehyde. The results are presented in Figure 3, and show that the reaction is largely complete within about two hours. Although it is evident that the reaction with 4.4 eq is faster than 2.1 eq, for both reactions the final concentration of benzaldehyde approaches to within 2% of the expected value, meaning that the formation of the AMP-benzylidene derivative is highly favorable relative to the reverse hydrolysis reaction.

Validation of Method for AMP in Samples of API

Based on a tentative specification limit of 30 ppm of AMP in the API, the linearity was tested in the range of about 15 to 190 ppm of AMP relative to API. The possibility of the API causing interference in the derivatization

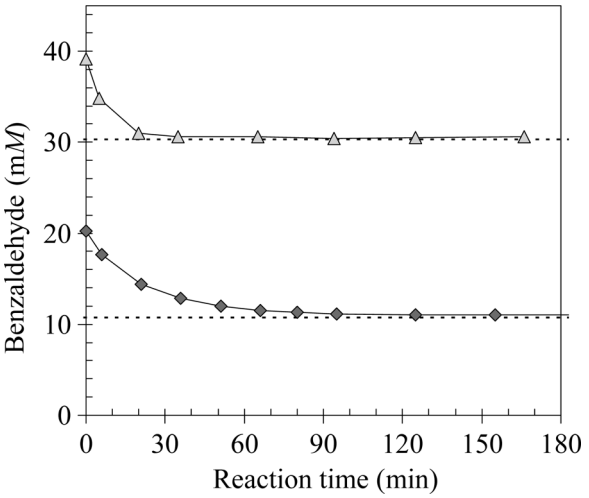

FIGURE 3 Change in concentration of benzaldehyde over time as reaction with AMP progresses. Triangles show reaction with 4.4 eq benzaldehyde (initial concentrations of 39.16 mM benzaldehyde and 8.85 mM AMP). Diamonds show reaction with 2.1 eq benzaldehyde (initial concentrations 20.25 mM benzaldehyde and 9.48 mM AMP). In each case, dotted line shows expected final concentration of benzaldehyde (30.31 and 10.77 mM, respectively).

reaction was also a concern. Therefore, spiked recovery tests were conducted at several levels, including the specification limit. The results are shown in Table 1, which shows that good linearity and recovery was obtained in the range tested. The stability of the derivative in solution was verified by reinjecting several samples 20 hours after the initial analysis, which in all

TABLE 1 Linearity, Recovery, and Precision of Method. Linearity of Derivative Peak Area in Preparations of AMP + Benzaldehyde, Based on Plot of Peak Area versus AMP Concentration (in Units of ppm, Relative to Nominal 0.5 mg/mL Concentration of API). Linearity Tested Across AMP Levels of 14.2, 28.5, 47.4, 94.8, 142, and 190 ppm. Spiked Recovery Tests are in Presence of API in Range of 28.5 to 190 ppm of AMP, and Stability of Samples for an Additional 20 Hours After the Initial HPLC Analysis. Precision Test Conducted by Determining S_{REL} for 6 Replicate Injections at Specification Level of 28.5 ppm

Linearity slope	62.96
Linearity intercept	−361.0
Linearity correlation (r^2)	0.9973
Recovery (%), 190 ppm	97.9
Recovery (%), 142 ppm	102.7
Recovery (%), 95.0 ppm (after additional 20 hr)	106.0 (104.9)
Recovery (%), 47.4 ppm (after additional 20 hr)	96.0 (97.6)
Recovery (%), 28.5 ppm (after additional 20 hr)	109.4 (97.3)
S_{REL} (n = 6) at 28.5 ppm level	8.2

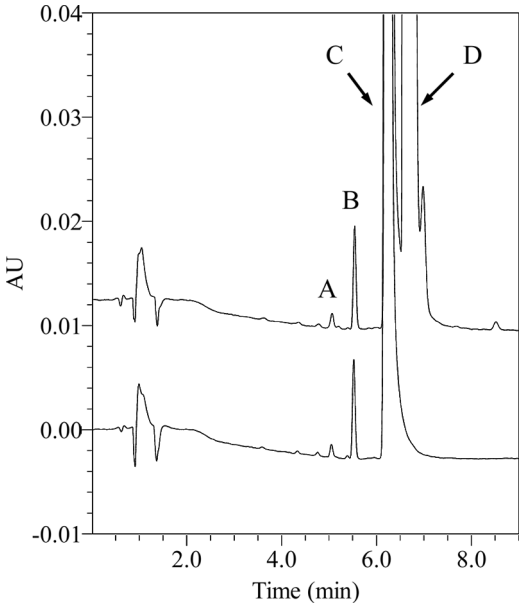

FIGURE 4 Example HPLC-UV chromatograms showing (lower trace) reference standard injection of AMP-benzaldehyde derivative (nominal 190 ppm), and (upper trace) sample of API spiked with 190 ppm AMP, showing separation of (A) benzylidene derivative, (B) benzoic acid impurity, (C) unreacted benzaldehyde, and (D) API. HPLC-UV conditions described in experimental section.

cases showed acceptable recovery values. Precision of the method was also determined to be acceptable when tested at the specification level, based on six replicate injections, which afforded a relative standard deviation below 10% (Table 1).

Example HPLC-UV chromatograms are shown in Figure 4. The AMP derivative peak was well separated from the API peak and the unreacted benzaldehyde peak. It was also separated from the benzoic acid impurity which is typically present in benzaldehyde reagent, presumably due to oxidation of benzaldehyde reagent itself or traces of benzyl alcohol in the benzaldehyde reagent.[12]

CONCLUSIONS

The reaction of aldehydes or ketones with organohydrazines to produce hydrazone derivatives has long been exploited for aldehyde and ketone analysis. Although the reaction is also potentially useful for analysis of organohydrazines, previous reports of the reaction being used in this manner are relatively rare. In this study, we demonstrate the utility of this approach for analysis of AMP, an organohydrazine type synthetic

byproduct, in samples of an API. Benzaldehyde was used as derivatizing reagent, and produced a stable AMP-benzylidene derivative in solution. The desired condensation reaction between benzaldehyde and AMP was shown to be strongly favored compared to the reverse hydrolysis reaction, based on HPLC-UV monitoring of benzaldehyde consumption. The AMP-benzylidene derivative was easily detected at 30 ppm level by HPLC-UV. The simplicity of the method was well suited for testing a relatively large number of samples in a short time frame using readily available HPLC-UV instrumentation. The method played a critical role during synthetic process development of the API, allowing synthetic chemists to optimize purification processes for the API during development and scale-up activities. Although, only one organohydrazine type compound was investigated in this study, the results suggest that other organohydrazine compounds may also be amenable to the same derivatization technique.

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