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Short communication

Catalytic precolumn derivatization of amikacin

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1. Introduction

Amikacin sulfate, a semi-synthetic aminoglycoside antibiotic derived from kanamycin, is indicated for the intravenous or intramuscular treatment of gram-negative bacteria. Since amikacin lacks any chromophore, it is often derivatized to form a UV absorbing species prior to HPLC analysis. Derivatization of aminoglycosides using 2,4,6-trinitrobenzenesulfonic acid (TNBS) was first reported by Benjamin for the spectrophotometric assay of these antibiotic drugs in serum and other biological fluids [1]. Quantitative HPLC determination of amikacin in pharmaceutical formulations using precolumn derivatization was first described in 1985 by Gambardella [2]. Gambardella reported the use of TNBS as a derivatization reagent in a pyridine–water mixed solvent for the precolumn derivatization of several aminoglycosides including Amikacin. Although Gambardella reported good precision for the assay method, the robustness of the assay with respect to the origin of pyridine used was not investigated.

An assay method based on Gambardella's procedure was developed in our laboratory in 1990 for the determination of amikacin in amikacin sulfate injection [unpublished]. This method is also similar to the method subsequently published in Supplement 6 of USP–XXII in 1992 [3]. The only significant difference between Gambardella's method, our method and the USP method are in the proportions of the TNBS reagent and pyridine used. Although the method developed here has been effective, it periodically showed some variability of results depending on the source of the pyridine used. In particular, severe fronting of the amikacin peak was also observed when certain lots of pyridine were used (Fig. 1). Peak fronting is usually indicative of incomplete derivatization and failure of the assay method. Examination of the USP method also occasionally shows peak fronting, especially when purified pyridines are

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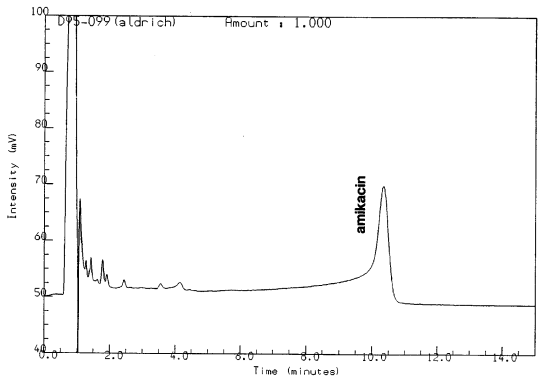

Fig. 1. Chromatogram of amikacin injection derivatized with high purity pyridine and without the use of a catalyst.

used in the procedure. Even when severe peak fronting is not observed, the %RSD of the peak area resulted from an uncatalyzed TNBS derivatization procedure can be as high as 5% with respect to pyridine. Although this degree of variability does not occur if the same lot of pyridine is used (Table 1), a higher degree of accuracy and precision is often needed for long term stability testing where it is not possible to use the same bottle of pyridine. Modification of the assay procedure through the use of 4-DMAP catalyst, addition of an effective quenching reagent, and equalization of sample matrix in the standard and sample improved the accuracy and precision significantly.

2. Experimental

2.1. Reagents

Amikacin Sulfate Injection, 250 mg ml^{-1}, was manufactured by DuPont Merck, Aguadilla, P.R. Amikacin (base) USP reference standard was from the U.S.P., Rockville, MD. Methanol and acetonitrile were HPLC grade. Liquid chromatography quality water was obtained by passing distilled water through a Milli-Q Water System purification unit (Millipore, Bedford, MA). Sulfuric acid, sodium citrate, 2,4,6-trinitrobenzenesulfonic acid, sodium hydroxide, pyridine, triflouroacetic acid (TFA), and potassium phosphate monobasic, anhydrous, were all reagent grade. 4-Dimethylaminopyridine (4-DMAP), 99%+ was from Aldrich, Milwaukee, WI.

2.2. Instrumentation

Method development was performed on a Waters® HPLC system equipped with a Waters® model 715 WISP, Waters® model 510 pump, Waters® model 680 gradient controller, and an Applied Biosystems model 759A absorbance detector. Derivatization was carried out in a Neslab® circulating water bath. Chromatographic data were collected on a Fisons® VG–multichrom data system. A summary of the HPLC conditions is listed in Table 2 and a typical chromatogram is shown in Fig. 2.

Table 1
Variability of the USP method with respect to pyridine[a]

Pyridine manufacturer	Lot number	% of label	% RSD[b]
J.T. Baker	H42614	103.4	0.28
J.T. Baker	F38616	100.1	0.44
J.T. Baker	D41610	105.5	0.47
Aldrich	07657DG	93.6	0.29
Burdick and Jackson	B1758	107.4	0.40
EM Science	34224440	104.6	0.06
Average		102.4	0.32
Variability with respect to pyridine (%RSD)		4.8	

[a] All assays were performed on the same day.
[b] Variability of the assay when the same lot of pyridine was used ($n = 2$).

Table 2
Summary of HPLC conditions

HPLC column	Zorbax® SB-C8, 15 cm × 4.6 mm i.d.
Mobile phase	45% of 0.02 M potassium phosphate, monobasic, 41% acetonitrile, and 14% methanol, adjusted for a final pH of 7.7 with 50% sodium hydroxide.
Flow rate	2.0 ml min^{-1}
Column temperature	Ambient
Injection volume	20 μl
Detection	UV at 350 nm

2.3. Preparation of standards and samples

Standard solutions were prepared from Amikacin USP reference standard with a use-as value of 92.0% at 0.90 mg ml^{-1}, 1.10 mg ml^{-1}, and 1.35 mg ml^{-1} in 0.4 mM sodium citrate buffer which had been adjusted to a pH of 4.5 with sulfuric acid. Amikacin Sulfate Injection samples were diluted in water to a concentration of 1.0 mg ml^{-1}.

2.4. Derivatization procedure

125 μl of each standard and sample solution were pipetted into separate 4-ml autosampler vials. 1.60 ml of pyridine, 500 μl of a 4-dimethylaminopyridine solution (0.9% w/v) and 500 μl of TNBS reagent (0.5% w/v) were then pipetted into each vial. The vials were capped with a Teflon-coated lid, shaken to mix, and heated at 75°C for 75 min. The solutions were allowed to cool to room temperature and then 500 μl of 20% triflouroacetic acid in acetonitrile were added to each vial. The solutions were capped, shaken to mix, and injected directly into the chromatograph. Since the primary concern for this investigation is to ensure that the derivatization reaction is driven to completion, the reaction condition of 75°C and 75 min was chosen. For routine analysis, a shorter reaction time may be adequate.

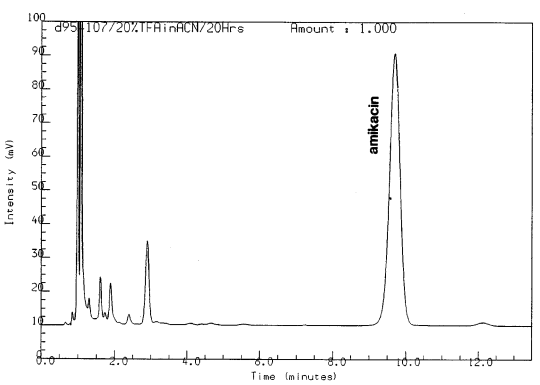

Fig. 2. Typical chromatogram from a 4-DMAP catalyzed derivatization procedure.

3. Results and discussion

3.1. Variability of the USP method with respect to pyridine

A stability sample (Lot BPN3938-73B, 3-yr, 25°C) and a sample from a commercial lot (Lot No. 4QV201-4) were tested with different lots of pyridine from different manufacturers. Pyridine was not distilled or further purified. Although the average precision (RSD) of the USP assay was at 0.32% when the same lot of pyridine was used, the variability with respect to pyridine was 4.8% when different lots of pyridine were used. Results for the variability of the USP assay are listed in Table 1.

3.2. Variability of the catalytic derivatization method

With the improved method, the precision of the assay with respect to different lots of pyridine improved to less than 2%. For Amikacin Injection lot number BPN3938-73B, the %RSD with respect to six lots of pyridine was 1.3%. For Amikacin Injection lot number 4QV201-4, the %RSD with respect to three lots of pyridine was 1.6%. The results also demonstrated day-to-day ruggedness since these catalytic derivatizations were performed on different days. Results are shown in Tables 3 and 4.

Table 3
Robustness of the catalyzed derivatization method with respect to pyridine (Amikacin Injection lot BPN3938-73B)

Pyridine manufacturer	Pyridine lot number	Assay date	% of label
J.T. Baker	H42614	3/10/95	99.0
J.T. Baker	H42614	3/15/95	100.0
J.T. Baker	F38616	3/20/95	98.6
J.T. Baker	D41610	3/10/95	99.0
Aldrich	07657DG	3/30/95	101.5
Burdick and Jackson	B1758	3/10/95	101.4
Average			99.9
Variability with respect to pyridine (%RSD)			1.3

3.3. Accuracy

To verify the accuracy of the revised assay, a recovery sample was prepared from a precisely weighed amount of USP amikacin (base) reference standard. To the reference standard solution, a 2:1 molar excess of sulfuric acid was added to convert the amikacin base to amikacin sulfate. Sodium citrate was added to simulate the sample matrix of a typical amikacin sulfate injection sample solution. The actual concentration, as determined by the amount of amikacin reference standard weighed, was at 101.9% of label. Table 5 shows the recovery of this sample on 4 different days with 6 different lots of pyridine from different suppliers. The pyridine ranged from reagent to GC grade. The theoretical value for the sample, based on the amount of USP amikacin weighed, was 101.9% of the labeled amount. The average % recovery was 99.1% with a %RSD of 1.1. The data are summarized in Table 5.

3.4. Sample solution stability

Samples and standards treated with trifluoroacetic acid showed no significant changes in peak area after 44 h. The %RSD for five injections injected within a 44 h period showed a relative standard deviation of less than one percent. This showed that the quenching agent was effective and the derivatized amikacin was stable in the quenched reaction mixture. Results are shown in Table 6.

3.5. Effect of the catalyst

The detail reaction mechanism between primary amines and trinitrobenzenesulfonic acid, along with structures of chemical intermediates, were reported by Means et al. [4]. The chemical structure of amikacin, which shows the amine functional groups on the molecule, can be found in the USP. The amino groups on amikacin act as nucleophiles for the nucleophilic aromatic substitution reaction between amikacin and TNBS. In this derivatization system, pyridine serves a dual role as a reagent to maintain a basic reaction environment for the nucleophilic substitution reaction and as a co-solvent for the non-polar amikacin–TNBS adduct.

In addition, most reagent grade pyridines are known to contain trace impurities such as picolines (methylpyridines) and lutidines (dimethylpyridines) [5]. These and other impurities in reagent grade pyridine may serve as a catalyst for this nucleophilic substitution reaction. Depending upon the source and purity of the pyridine, there may be more or less of these catalysts present since a highly purified grade of pyridine is likely to contain a very low concentration of picolines and other substituted pyridines. Our experience showed that when highly purified pyridine (e.g. distilled in glass under nitrogen grade) is used in an uncatalyzed TNBS derivatization reaction, low recovery and peak fronting occurs.

The addition of 4-dimethylaminopyridine, a known hypernucleophilic catalyst for nucleophilic aromatic substitution [6], assures the presence of a

Table 4
Robustness of the catalyzed derivatization method with respect to pyridine (Amikacin Injection Lot No. 4QV201-4)

Pyridine manufacturer	Pyridine lot number	Assay date	% of label
J.T. Baker	H42614	3/15/95	103.8
J.T. Baker	F38616	3/20/95	100.9
Aldrich	07657DG	3/20/95	103.8
Average			102.8
Variability with respect to pyridine (%RSD)			1.6

consistent amount of catalyst regardless of the source of pyridine used. When a consistent amount of catalyst is present, complete derivatization of amikacin is assured. Our results showed that when 4-DMAP is added as a catalyst, peak fronting is eliminated regardless of the source of the pyridine used. Lack of peak-fronting usually indicates complete derivatization of amikacin by TNBS.

Even though HPLC peak fronting was eliminated when the 4-DMAP catalyst was used, the accuracy and precision of the assay procedure were still less than adequate. The inaccuracies were traced to two other sources of error: dissimilar sample matrix and inadequate reaction quenching.

3.6. Equalization of standard and sample matrices

Sodium citrate, an excipient in the Amikacin Sulfate Injection, appears to enhance the degree of derivatization. The percent recovery increased in the presence of sodium citrate. Sodium citrate was found to increase the peak area by as much as 7–15% depending on the lot and source of the pyridine used during the derivatization. To ensure that amikacin in the standard and sample solution were derivatized under the same condition, the Amikacin reference standard was dissolved in a solution containing the same citrate concentration as the drug product. In addition, one normal sulfuric acid was used to adjust the pH of the amikacin base standard to 4.5, the same pH as Amikacin Injection. The other excipient, sodium metabisulfite, was found to have no effect on the derivatization reaction.

3.7. Addition of an effective quenching reagent

Previous sample solution stability studies have shown that the sample-peak area continued to change if the sample was reinjected after it was derivatized. Addition of trifluoroacetic acid after chemical derivatization acidifies the reaction condition and effectively stops any further nucleophilic substitution reactions. Post-derivatization

Table 5
Accuracy of the catalytic derivatization method using different sources of pyridine

Pyridine manufacturer	Pyridine lot number	Assay date	% of label	% recovery
J.T. Baker	H42614	3/10/95	101.0	99.1
Burdick and Jackson	B1758	3/10/95	102.2	100.1
J.T. Baker	D41610	3/10/95	99.7	97.8
J.T. Baker	H42614	3/15/95	101.2	99.3
Aldrich	07657DG	3/20/95	102.3	100.4
J.T. Baker	F38616	3/20/95	99.8	97.4
Average			101.0	99.1
Variability with respect to pyridine (%RSD)			1.1	1.1

Table 6
Sample solution stability after quenching with TFA

Time	% of label (sample)[a]	% of label (standard)
T(0)	106.27	102.19
11 hours	106.07	101.59
22 hours	104.97	101.77
33 hours	105.01	101.49
44 hours	104.75	101.66
% Decrease in 44 hours	1.40	0.52

[a] Sample formulated at approximately 105% of label.

addition of weaker organic acids such as citric acid was attempted but weaker organic acids were not strong enough to stop the reaction. Strong inorganic acids, such as hydrochloric acid and sulfuric acid, decomposed the TNBS-amikacin adduct.

4. Conclusion

On the basis of the results presented above, the derivatization procedure was shown to be no longer sensitive to the origin of the pyridine used. By modifying the standard preparation procedure and the use of an effective quenching reagent, the accuracy and precision of the assay were significantly improved. The results from the robustness and recovery studies were accurate and precise. The revised assay is linear and the sample solution is stable for more than 4 days.

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