

Uncovering Deficiencies in Mass Balance Using HPLC with Chemiluminescence Nitrogen-Specific Detection



The author describes using high performance liquid chromatography (HPLC) with chemiluminescence nitrogen-specific detection and amperometric detection to determine organic impurities in a tedisamil dihydrochloride drug substance. The results obtained confirm the validity of the method and its ability to clarify deficiencies in mass balance during forced degradation. The HPLC method with amperometric detection was acceptable as a stability-indicating method for routine stability testing of the drug substance and the dosage form. The chemiluminescence nitrogen-specific detection method, however, provided reliable, more-accurate results that revealed markedly less deficiency in mass balance.

Stability testing requires powerful and efficient chromatographic methods in terms of their selectivity, limits of quantitation, and specificity. It can be accomplished if no gap in mass balance occurs during degradation, when quantifying the main compound and the degradation products formed. To establish mass balance, the real response factors — the ratio of the mass injected versus the peak area measured — for all impurities must be known; however, this situation often is not the case during early method development and frequently is a challenging task for validation in the later stages of method development. If analysts assume identical response factors for unknowns and the main compound, they could obtain misleading and inaccurate results (1). Consequently, the guidelines suggest that mass balance is recommended, although not always possible (2).

Tedisamil from Solvay Pharmaceuticals is an antiarrhythmic potassium channel-blocking agent currently in clinical development. Because of the drug substance's lack of chromophores, organic impurities and degradation products in the drug substance and in its formulations currently are determined by isocratic reversed-phase high performance liquid chromatography (HPLC)

with amperometric detection. It has been shown elsewhere that the selectivity of this method is sufficient to separate the potential organic impurities; that is, synthesis by-products and a specified degradation product from the drug substance (3).

The objective of this article is to demonstrate that this method's suitability for stability testing can be evaluated easily by performing HPLC with chemiluminescence nitrogen-specific detection as a reference method (4).

Nitrogen-specific detection first was used in gas chromatography for the determination of nitrosamines (5). Coupling a nitrogen-specific detector with an HPLC system required some modifications, but it proved to be a useful instrument for quantifying peptides without the need for prior derivatization (6). Researchers have described applications for biochemical analysis (7,8) and combinatorial chemistry (9). Until now, the use of chemiluminescence nitrogen-specific detection for method validation and impurity profiling (10), including complete mass balancing for pharmaceutical applications, has not yet been published in the literature, to my knowledge.

The principle of operation for the nitrogen-specific detector used begins with the complete high-temperature oxidation of

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the entire sample. A nebulizer supplied with argon and oxygen sprays the mobile phase into a pyrolysis tube positioned in an oven, which is heated to 1050 °C. As each component is eluted from the column, nitrous oxide forms and is dried and transferred to a reaction chamber where it is combined with ozone, produced by an onboard ozone generator, to form NO_2^* (nitrogen oxide in the excited state). As the excited state decays to the ground state, light is emitted and detected at specific wavelengths by a photomultiplier tube. This chemiluminescent emission is specific for nitrogen and is proportional to the amount of nitrogen in each compound.

Analysts most commonly inject stressed alkaline, acidic, or oxidative solutions of drug substances to evaluate the stability-indicating properties of chromatographic methods. For my investigations, I analyzed samples from stressed solutions in parallel by HPLC with amperometric and chemiluminescence nitrogen-specific detection. The area percentages determined by chemiluminescence nitrogen-specific detection are reliable as reference values that require no correction by relative response factors. This reliability is due to the potential degradation products, which differ only slightly by their molecular mass. Additionally, the bispidine cyclus with two nitrogens contained remains unaffected during degradation, so the molar percentages obtained by this method are similar to weight percentages (Figure 1).

I calculated the mass balance for both methods then compared them, taking into consideration the amount of drug substance determined and the sum of all degradation products. I also analyzed the solutions obtained by forced degradation using flow

injection coupled with the chemiluminescence nitrogen detector to compare the total amount of nitrogen with the theoretical amount that was supposed to be present in the solution. This amount should have corresponded with the sum of all impurities and the drug substance, if no compounds adhered to the stationary phase, and with the amount originally present, if no volatile nitrogen-containing compounds formed during degradation.

The compounds from HPLC–chemiluminescence nitrogen-specific detection were separated within the analytical run by column switching and reinjected for analysis by HPLC–amperometric detection to verify which compounds from the less selective detection mode (chemiluminescence nitrogen-specific detection) could be detected by amperometric detection. In close cooperation with my company's research analysis team, my colleagues and I determined the molecular masses for these compounds by HPLC–mass spectrometry (MS) and identified them by liquid chromatography–nuclear magnetic resonance spectroscopy (LC–NMR), which is described elsewhere (11).

Experimental

Chemicals and reagents: I obtained acetic acid, acetonitrile, methanol, sodium dihydrogen phosphate, and hydrogen peroxide from Merck KGaA (Darmstadt, Germany). The phosphoric acid was from Riedel-de Haen (Seelze, Germany), and the trifluoroacetic acid was from Solvay Fluor & Derivatives (Hannover, Germany).

I used tedisamil dihydrochloride, batch A9-4/M (Solvay Pharmaceuticals), to prepare the solutions, which were subjected to

hydrolytic or oxidative stressing storage conditions, as outlined below in the "Preparation of test solutions" section.

The sodium hydroxide solution was taken from Fixanal 0.1 N sodium hydroxide solution ampuls (Merck). I prepared the hydrochloric acid from Fixanal 1 N hydrochloric acid solution ampuls (Merck).

Equipment: HPLC–electrochemical detection: The HPLC system comprised a model L 6200 A pump, a model AS 2000 A autosampler, and a 10- μL sample loop (all from Merck–Hitachi, Darmstadt, Germany) and a BAS LC-4C amperometric detector (Bioanalytical Systems, Inc., West Lafayette, Indiana). The amperometric detector was coupled with a PC, which used System Manager D-7000 software (Merck–Hitachi), through a Merck interface.

The column was thermostated in a Gynkotec Peltier column oven (Gynkotec, Germering, Germany). Analytes were separated before amperometric detection using a 150 mm \times 4.6 mm, 5- μm d_p Zorbax 80-5 SB-CN cyanopropylsilyl column (Agilent Technologies GmbH, Waldbronn, Germany). The mobile phase was 580:50:370:1 (v/v/v/v) water–acetic acid–acetonitrile–phosphoric acid at a flow rate of 1.0 mL/min. The pH of the mobile phase was adjusted to pH 5.0. The amount injected of the tedisamil dihydrochloride sample was 5 μg .

Electrochemical detection was performed using a glassy carbon electrode with the potential set at +1150 mV.

I evaluated the amount of each by-product by calculating the percentage of the peak area to the total sum of peak areas; I used no relative response factors for recalculation.

HPLC–chemiluminescence nitrogen-specific detection: HPLC with chemiluminescence nitrogen-specific detection was performed using the same HPLC equipment described above with a model 8060 chemiluminescence nitrogen-specific detection detector (Antek Instruments, Houston, Texas) instead of the electrochemical detector. The temperature of the column was controlled by a Gynkotec column oven at 25 °C.

I performed gradient separations using a 125 mm \times 3 mm, 3- μm d_p ProntoSIL 120-3 ODSAQ stainless steel analytical column (Bischoff Chromatography, Leonberg, Germany). I added 0.5 mL of trifluoroacetic acid to each 1000 mL of water and methanol during mobile-phase preparation. The 0.4-mL/min flow rate was adjusted isocratically by running 15:85 (v/v) methanol–water with 0.05% trifluoroacetic acid for

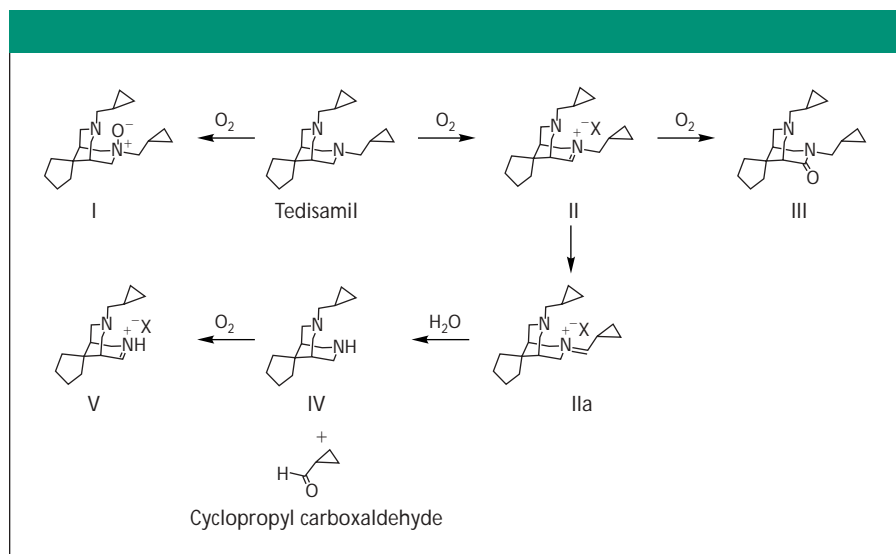


Figure 1: Proposed degradation mechanism of tedisamil dihydrochloride under alkaline and oxidative conditions.

5 min and then starting a gradient to 90:10 (v/v) methanol–water with 0.05% trifluoroacetic acid in 30 min. Before entering the detector, the flow was split using a tee and suitable PEEK capillaries to direct 0.15 mL/min of the flow to the nebulizer and 0.25 mL/min to waste.

The detector was operated with an oven temperature of 1050 °C with argon and oxygen flow rates adjusted to 180 and 135 mL/min, respectively. The pressure measured at the nebulizer was 20 psi. The ozone flow was adjusted to 25.6 mL/min, and the high voltage of the photomultiplier tube was adjusted to –775 V. The photomultiplier tube was chilled to –10 °C.

I performed flow injection using water with 0.05% trifluoroacetic acid as the mobile phase. The column was replaced by a stainless steel junction. The peaks were isolated after the split by a Valco E-C6W electrically actuated, six-way valve (Valco Instruments Co. Inc., Houston, Texas). I injected 50 μ L of the collected fractions or solutions of degraded tedisamil dihydrochloride diluted 1:5 (v/v) with mobile phase into the HPLC system for analysis with electrochemical detection.

I validated the method with respect to selectivity using four by-products (10) and was able to separate the degradation products depicted in Figure 1. The precision for quantification of the main compound was 1.68%, calculated from the assays obtained for six preparations at a concentration of 1 mg/mL with a single injection for each. The precision for impurities (six injections) was 4.5% at a concentration of 1 μ g/mL. I confirmed the linearity within a working range of 0.002–1 mg/mL and calculated the limit of quantitation, based upon a signal-to-noise ratio of 10:1, to be 0.3–0.6 μ g/mL. Equimolarity was proven for three by-products and degradation products that differed significantly in structure. I measured 101.0–104.2% of calculated peak areas in the validation experiments.

HPLC–UV: To quantify tedisamil dihydrochloride, I used an HPLC method that was proven selective for the separation of tedisamil dihydrochloride from the degradation products and was fully validated according to International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (Geneva, Switzerland) guidelines. I performed HPLC with UV detection using the same HPLC equipment as described above but with a model L-4250 UV–vis detector (Merck–Hitachi) in place of the electrochemical detector. The detection wavelength was adjusted to 205 nm.

The temperature of the column was controlled by a Gynkotek column oven at 30 °C. I performed isocratic separation using a 125 mm \times 3 mm, 3- μ m d_p ProntoSIL 120-3 ODSAQ stainless steel analytical column. The autosampler was equipped with a 10- μ L sample loop. The degraded solutions were injected after dilution to 1:5 (v/v). The mobile phase was 60% acetonitrile by volume and phosphate buffer that contained 6.9 g/L of sodium dihydrogen phosphate monohydrate with the pH adjusted to 2.3 using with phosphoric acid. The flow rate was 1.0 mL/min.

Preparation of test solutions: *Degradation in alkaline medium:* I weighed 125.0 mg of tedisamil dihydrochloride into each of two brown screw-cap glass bottles. After adding 25.0 mL of 0.1 N sodium hydroxide solution, I closed the bottles and stored them at 50 °C for 24 h. After cooling the bottles to ambient temperature, I stopped the reaction by adding 2.5 mL of 1 N hydrochloric acid and analyzed the solutions.

Degradation in oxidative medium: I also weighed 125.0 mg of tedisamil dihydrochloride into each of two brown screw-cap glass bottles. After adding 25.0 mL of 1% hydrogen peroxide solution, I closed the bottles and stored them at 50 °C for 22 h.

Results and Discussion

Degradation mechanism: The degradation mechanism was clarified by LC–NMR and MS in parallel to the experiments described herein (Figure 1). The amine oxides and imines and iminium derivatives are the beginning products of the oxidation chain formed by oxidizing tertiary amines such as tedisamil dihydrochloride with hydrogen peroxide or air under alkaline catalysis, respectively. Both radical and ionic mechanisms are involved.

The oxidation of tedisamil dihydrochloride with dilute hydrogen peroxide (1%) at 50 °C furnished the tedisamil mono *N*-oxide (I) and the mono iminium derivative of tedisamil (II) as main products, whereas the oxidation with air in the presence of 0.1 N sodium hydroxide at 50 °C produced follow-up oxidation and hydrolysis products other than the two main oxidation products.

The lactam (III) was obtained by oxidation of compound II by the corresponding α -hydroxy amine, which was in equilibrium with the iminium. The prototropic rearrangement of the endocyclic double bond of compound II under basic catalysis formed the thermodynamically less stable product with an exocyclic double bond

(IIa). The resulting isomeric iminium salt was hydrolyzed easily to yield cyclopropyl carboxaldehyde and the dealkylated tedisamil (IV). The secondary amine (IV) was dehydrogenated to yield the imino compound (V).

Degradation in alkaline and oxidative medium: *Quantitative results:* The results obtained for the amounts of degradation products by HPLC–ECD and HPLC–chemiluminescence nitrogen-specific detection, respectively, are listed in Tables I and II as relative area percentage with the sum of all peak areas as 100%. Figures 2 and 3 show the corresponding chromatograms for alkaline degradation obtained by both methods. Figures 4 and 5 illustrate the corresponding chromatograms for oxidative degradation obtained by both methods.

I isolated the major degradation products, which correspond to the peaks numbered in the chromatogram, from HPLC–chemiluminescence nitrogen-specific detection by switching the valve in the waste line to collect definite fractions of the effluent during detection. These fractions were reinjected into the HPLC system with amperometric detection to identify the corresponding peaks. The identification of compounds was performed based upon retention times and masses determined by LC–MS.

Peaks that could be associated with the same degradation product were numbered

identically in both chromatograms and are listed in Tables III and IV. No corresponding peaks were detectable for peak numbers 7, 8, 9, 10, 14, 15, and 16 injected into the HPLC–electrochemical detection system, most likely because of the low amounts formed by alkaline degradation. Remarkably, the compound corresponding with the prominent peak number 11, which is one of the main degradation products detected by HPLC–chemiluminescence nitrogen-specific detection, was undetectable by amperometric detection. No corresponding peak was detectable by amperometric detection for peak number 13, which was obtained after oxidative degradation and injection into the HPLC–electrochemical detection system.

Mass balance: Tables III and IV list the concentration of tedisamil dihydrochloride determined by HPLC–chemiluminescence nitrogen-specific detection calculated as a percentage related to the theoretical concentration of the drug substance before degradation and the sum of degradation products as relative area percentage, as well as the total amount determined by flow injection. I determined this amount by external standardization using a standard solution of tedisamil dihydrochloride of known concentration. Again, the initial concentration of tedisamil dihydrochloride before degradation was defined as 100%.

Table I: Relative retention times and area percentages for peaks isolated after alkaline degradation

Peak Number	Compound*	HPLC–Chemiluminescence Nitrogen-Specific Detection		HPLC–Electrochemical Detection	
		Relative Retention Time	Area (%)	Relative Retention Time	Area (%)
5	Unknown	0.15	4.88	0.20	4.47
6	II	0.18	4.26	0.46	4.06
12	IV	0.70	2.92	0.38	2.46
13	III	0.82	4.32	0.25	2.48
11	I	0.63	7.60	—	—

*Compound structures are shown in Figure 1.

Table II: Relative retention times and area percentages for peaks isolated after oxidative degradation

Peak Number	Compound*	HPLC–Chemiluminescence Nitrogen-Specific Detection		HPLC–Electrochemical Detection	
		Relative Retention Time	Area (%)	Relative Retention Time	Area (%)
4	II	0.16	7.2	0.47	7.8
13	I	0.60	29.4	—	—
14	Unknown	0.72	0.61	0.38	0.41
15	Unknown	0.81	1.3	0.4	8.0
16	Unknown	0.83	4.6	—	—

*Compound structures are shown in Figure 1.

I performed all calculations irrespective of the molecular weights or relative response factors of the individual degradation products. The amounts determined by HPLC–chemiluminescence nitrogen-specific detec-

tion, therefore, are relative area percentages and molar percentages.

Evaluation: I found a deficiency in mass balance for HPLC coupled with amperometric detection; however, HPLC–chemilu-

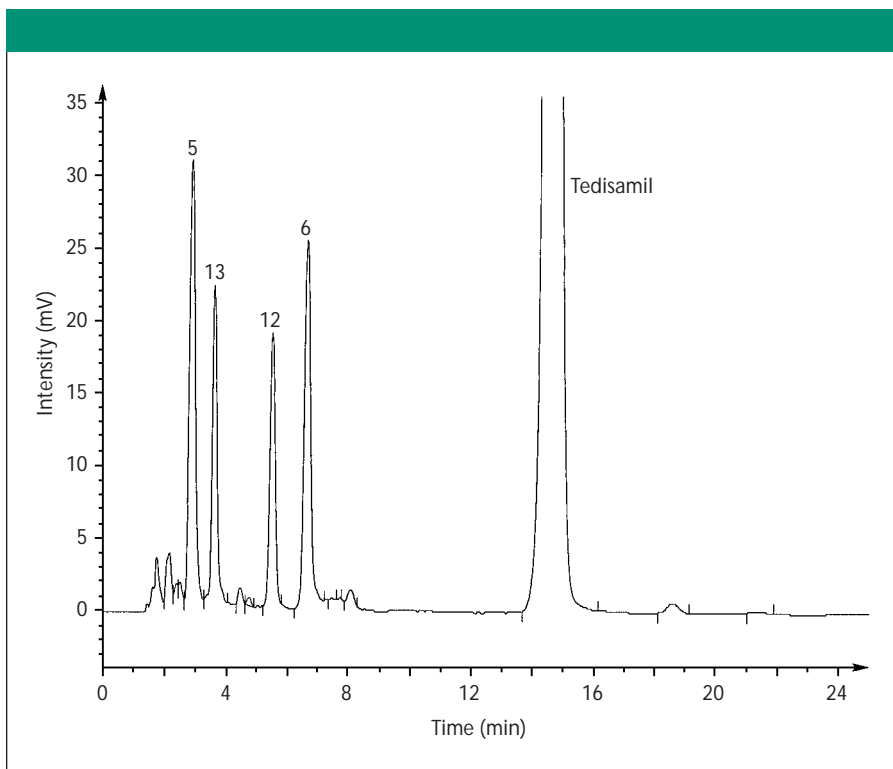


Figure 2: HPLC–electrochemical detection chromatogram obtained after alkaline degradation of tedisamil dihydrochloride. The fractions that were isolated from chemiluminescence nitrogen-specific detection and reinjected are numbered.

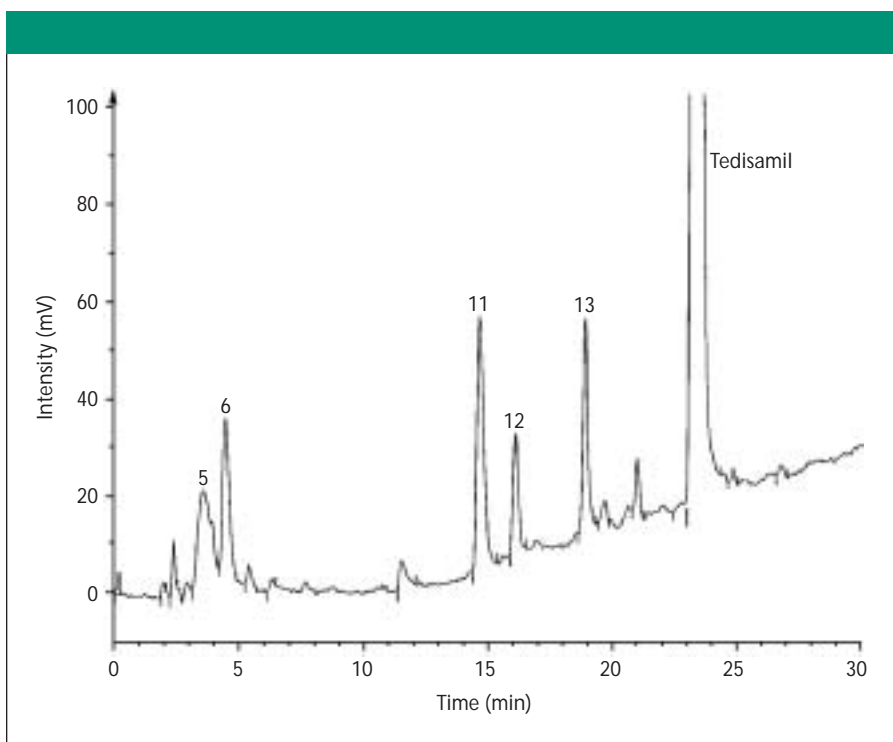


Figure 3: HPLC–chemiluminescence nitrogen-specific detection chromatogram obtained after alkaline degradation of tedisamil dihydrochloride. The fractions that were isolated are numbered.

minescence nitrogen-specific detection exhibited no significant losses due to chromatography or detection. The total amount determined by flow injection, which corresponds to the total nitrogen present in solution and the total sum of the assay (HPLC–UV), differed by only 4% from the sum of all degradation products for alkaline degradation. This difference is within the analytical precision standards that all peaks should be eluted within the run time of the HPLC–chemiluminescence nitrogen-specific detection gradient. The 91.6% amount, which near the theoretical expectation (100%), indicates that no losses occurred because of late eluted, volatile, or slightly soluble nitrogen-containing compounds that could have formed during degradation. The approximate 14% deficiency observed with the HPLC–electrochemical detection system can be ascribed primarily to fraction 11, which was undetectable by amperometric detection. I also found a significant difference in the amounts for peak number 13 (4.21 versus 2.48%) as determined by chemiluminescence nitrogen-specific detection and electrochemical detection, respectively.

I found a deficiency in mass balance of approximately 41% for oxidative degradation by HPLC with amperometric detection. The difference between the total amount determined by flow injection versus the sum of the assay and all degradation products determined by HPLC–chemiluminescence nitrogen-specific detection was only 9%, which is within the analytical precision standards. The 93.3% amount, which is near the expected 100%, indicates that no losses of nitrogen-containing compounds occurred during degradation. This difference compared with HPLC–electrochemical detection again was caused mainly by peak number 13, which was undetectable by amperometric detection. This fraction was identical to peak number 11 from alkaline degradation, and it was identified as the *N*-oxide of tedisamil dihydrochloride. Peak number 4 was identified by LC–MS and LC–NMR as the degradation product II.

Conclusion

The routine method used for the stability testing of tedisamil dihydrochloride and its final dosage form is based upon reversed-phase HPLC with amperometric detection. I used chemiluminescence nitrogen-specific detection as a reference method after flow injection and separation by reversed-phase HPLC. This method detects all compounds containing nitrogen and therefore theoretic-

cally covers all degradation products. Calculating the mass balance could prove this ability. The relative area percentages of degradation products detected were not corrected by relative response factors, assuming similar molecular masses for tedisamil and

its degradation products. Additionally, the degradation products retained the same number of nitrogen atoms. Deficiencies in mass balance for the amperometric detection occurred for degradation in the hydrogen peroxide solution and the dilute sodium

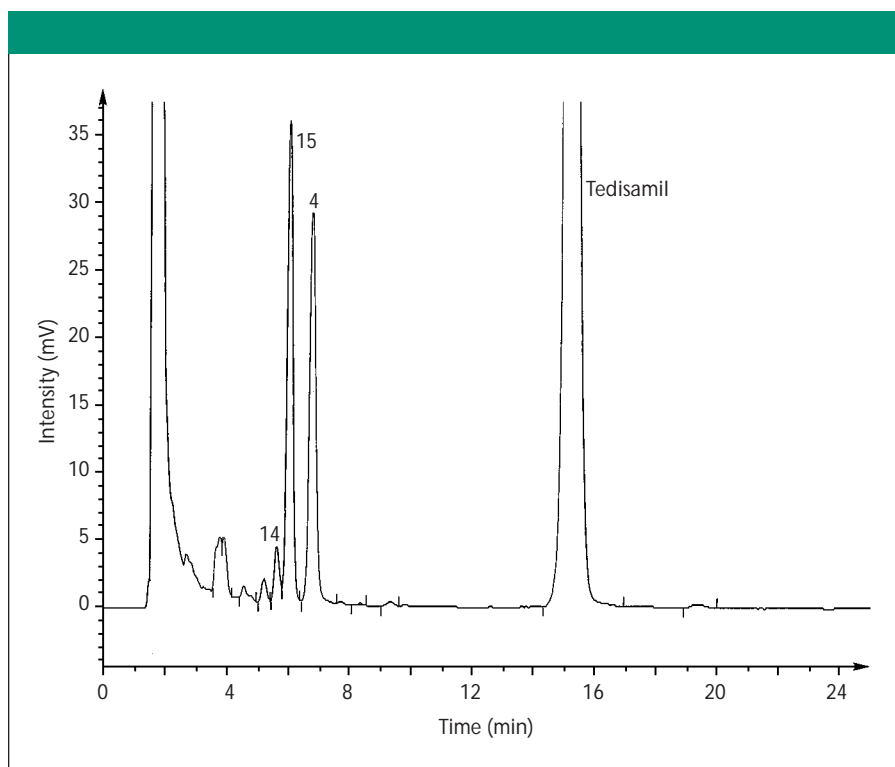


Figure 4: HPLC–electrochemical detection chromatogram obtained after oxidative degradation of tedisamil dihydrochloride.

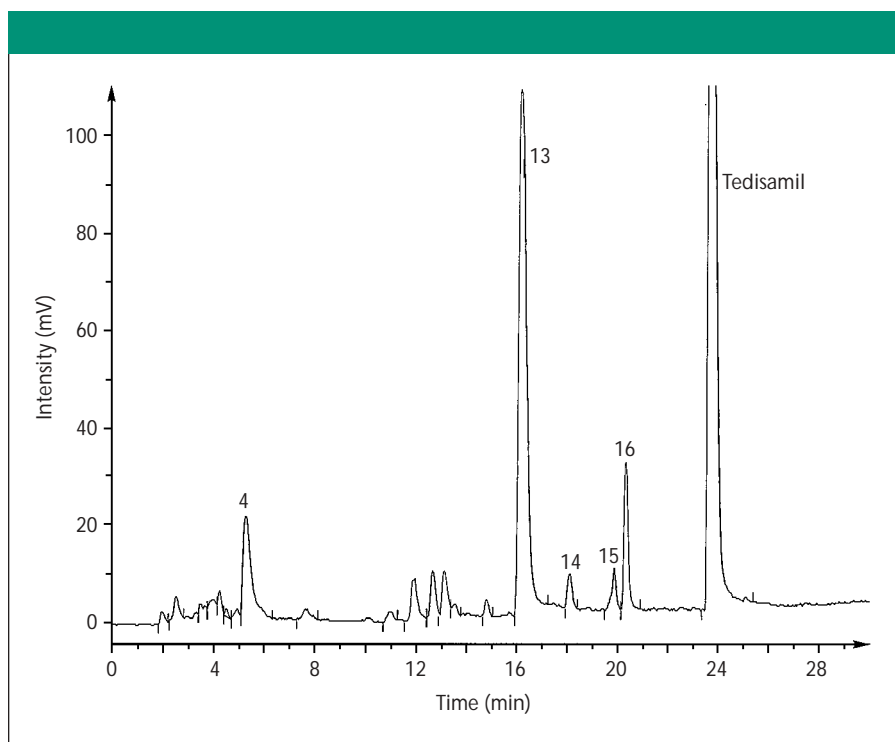


Figure 5: HPLC–chemiluminescence nitrogen-specific detection chromatogram obtained after oxidative degradation of tedisamil dihydrochloride.

Table III: Results tabulated for the calculation of the mass balance for alkaline degradation*

	HPLC-UV Detection	HPLC- Electrochemical Detection	HPLC-Chemiluminescence Nitrogen-Specific Detection
Assay (%)	59.1	—	—
Sum of degradation products (area %)	—	14.8	28.1
Sum of assay and degradation products (%)	—	73.9	87.2

*The assay was determined by a stability-indicating HPLC-UV method, and the sum of degradation products was determined by both methods. The total amount determined by coupling flow injection with chemiluminescence nitrogen-specific detection was 91.6%. The assay percentage is relative to the initial concentration of tedisamil dihydrochloride in solution.

Table IV: Results tabulated for calculation of the mass balance after oxidative degradation*

	HPLC-UV Detection	HPLC- Electrochemical Detection	HPLC-Chemiluminescence Nitrogen-Specific Detection
Assay (%)	40.0	—	—
Sum of degradation products (area percentage)	—	19.0	53.3
Sum of assay and degradation products (%)	—	59.0	93.3

*The determinations and calculations comply with Table I. The total amount determined by coupling flow injection with chemiluminescence nitrogen-specific detection was 102.3%.

hydroxide solution. These differences in mass balance were caused primarily by the *N*-oxide of tedisamil, which was undetectable by amperometric detection. I found a significantly different response factor for the degradation product III, the lactam. A correlation between both methods was confirmed for all other degradation products.

The experiments demonstrated that coupling HPLC with chemiluminescence nitrogen-specific detection in combination with LC-MS or other hyphenated techniques is a powerful approach to elucidate degradation mechanisms and clarify deficiencies in mass balance that could occur during stress and stability testing for all nitrogen-containing compounds. Separations can be achieved mostly by adaptation to systems that are compatible with chemiluminescence nitrogen-specific detection, although mobile phases are restricted to mixtures of methanol and water or alcohol and water with additional volatile modifiers.

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