

# Chemiluminescence Nitrogen-Specific Detection — A New Strategy in Analytical Development

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## Introduction

Quantification of impurities is one of the most meaningful and challenging tasks in pharmaceutical analysis. Requirements are defined in the International Conference on Harmonization (ICH) guidelines, including the corresponding limit of detection and the levels for reporting, identification and qualification (Table 1) (1). To rely on a sound rationale for quantification of impurities, however, the real response factor must also be known (i.e., the dependence of the signal size on the mass injected). This is the most important prerequisite for establishing the mass balance of degraded compounds, which is recommended though not always possible (2).

As a typical first approach, identical response factors are assumed for the impurities and the main compound, expressing the content of each impurity in area per cent. Subsequent steps would involve isolation and identification of the impurities above the limit of 0.1%, synthesis of reference standards and characterization by a number of different methods including the combination of a selective (e.g., chromatographic) method and an absolute method to demonstrate plausibility of the content defined (Table 2). The response factors and accuracy of the method would finally be verified by injection of the active pharmaceutical ingredient spiked with known amounts of the impurities or degradants.

This is a time-consuming and expensive procedure that may fail in some instances for which isolation, identification or synthesis of an impurity are not possible or will not be performed because of the amounts determined (expressed as area per cent) fulfilling ICH requirements.

Nitrogen-specific detection was first introduced to determine nitrosamines after separation by gas chromatography (3, 4). By coupling with high performance liquid chromatography (HPLC), which required some modifications, chemiluminescence nitrogen detection (CLND) was mainly used for the detection of peptides without the need for derivatization (5, 6). Coupling of supercritical fluid chromatography to CLND was successfully performed in some instances (7) (e.g., for detection of aza-steroids (8)). The principle of operation for the CLND nitrogen-specific detector used in our experiments begins with the complete high-temperature oxidation of the entire sample matrix as illustrated in Table 3. A nebulizer supplied with argon and oxygen is used to spray the mobile phase into a pyrolysis tube positioned in an oven, which is heated to 1050 °C. As each component elutes from the column, the NO formed is combined with ozone, produced by an on-board ozone generator, to form NO<sub>2</sub>\* (nitrogen oxide in the excited state). The NO<sub>2</sub>\* formed is dried and transferred to a reaction chamber. As the excited state decays to the ground state, a quantum of light is emitted and detected at specific wavelengths, by a photomultiplier tube (PMT). This chemiluminescent emission is specific for nitrogen and should be proportional to the amount of nitrogen in the isolated compound. To summarize, the main advantages of CLND are

- specificity for nitrogen-containing compounds

- selectivity to be tuned by chromatographic method
- equimolar response.

For method development suitable for CLND, the following requirements and restrictions have, however, to be respected:

- The mobile phase must not contain acetonitrile or any other compound containing nitrogen.
- Non-volatile buffers are to be avoided.
- The flow-rate must be less than 0.2 mL/min or split, if it is not possible to adjust it, using 2 mm columns, for example.
- The nitrogen content of the compounds should be known in order to estimate the expected limits of detection/quantification.

Tedisamil (Solvay Pharmaceuticals GmbH, Hannover, Germany) is a potassium channel blocking agent. The molecular weight calculated for the dihydrochloride is 361.4 (Figure 1). Because of the lack of a chromophore, determination of impurities is a challenge, keeping in mind the requirements of ICH for the limit of quantification, resulting from the levels given for reporting, identification and

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**Table 1:** Limits Defined in the ICH Guideline Q3B for Identification, Reporting and Qualification of Impurities for Different Daily Doses.

### ICH Guideline Q3B

#### Impurities in New Medicinal Products

Reporting	<1 g	0.10%
	>1 g	0.05%
Identification	<1 mg	1.00%
	1–10 mg	0.50%
	>10 mg–2 g	0.20%
Qualification	<10 mg	1.00%
	10–100 mg	0.50%
	100 mg–2 g	0.20%
	>2 g	0.10%

T2

**Table 2:** Steps to be Performed for Characterization of a New Impurity.

#### Characterization of a New Impurity

- Isolation
- Identification
- Synthesis
- Identification (NMR, MS, UV, IR, elemental etc.)
- Organic purity testing (GC, LC, CE, NMR etc.)
- Anorganic purity testing (sulfated ash, water etc.)
- Volatile impurities
- Definition of content by 100% method
- Confirming content by absolute method

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**Table 3:** Series of Reactions Producing Chemiluminescence for Nitrogen-Containing Compounds.

- $R-N + R-H + O_2 \xrightarrow{1050^\circ C} CO_2 + H_2O + NO + MO_x$
- $NO + O_3 \rightarrow NO_2^* + O_2$
- $NO_2^* \rightarrow NO_2 + hv$

qualification. Additionally, the potential differences in response factors may vary dramatically for ultraviolet detection when certain functional groups are introduced during synthesis or degradation (9). Tedisamil dihydrochloride and most of the potential by-products are electrochemically active and can, therefore, be detected by the coupling of electrochemical detection to HPLC. Degradation of the drug substance leads to formation of the iminium compound as the main degradation product (Figure 1). The method was specific for stability testing, but relative response factors were still questionable for the degradation products. Following the usual strategy for exact quantification and identity confirmation, synthesis of the degradation product was initiated after it had been identified from the degraded drug product.

The synthesis always produced very low yields of an impure and unstable product. Characterization of this material by common techniques was therefore impossible because no absolute method was available to confirm the content of the main

compound in the presence of a great number of partially unknown impurities.

If the degradation product could be quantified (e.g., as molar per cent) in a single chromatographic run using tedisamil dihydrochloride as an internal reference standard, then isolation and characterization of the by-product should no longer be necessary. Because of equimolarity, CLND was therefore considered to be the most suitable technique for obtaining reliable results that could be directly correlated to electrochemical detection by injection of the same solution.

A gradient chromatographic method was developed for coupling to CLND, based on the requirements outlined above. Selectivity was optimized with regard to the known potential by-products.

### Experimental

Tedisamil dihydrochloride and its main degradation product were manufactured in the Department of Pilot Synthesis Research of Solvay Pharmaceuticals GmbH (Hannover, Germany).

### Chromatography:

**Stationary phase:** A 125 × 3 mm stainless steel column was used, filled with Prontosil ODSAQ 3 μm, supplied by Bischoff Chromatography (Leonberg, Germany).

**Equipment:** The HPLC equipment used was a Merck Hitachi 6000 system (Merck,

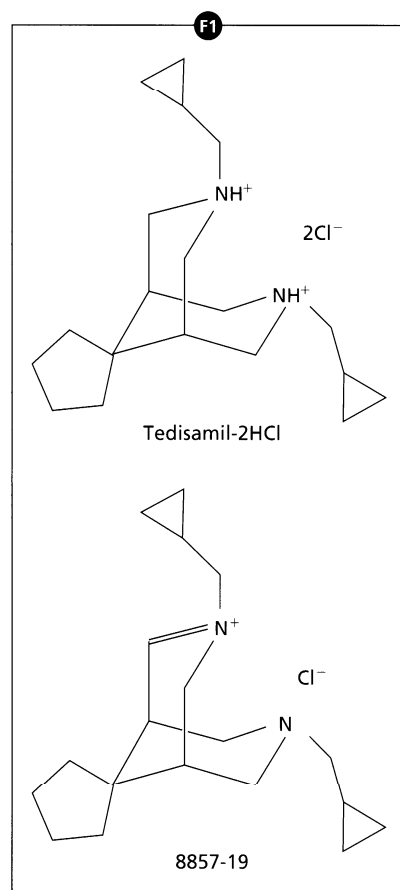
Darmstadt, Germany). The detector was an Antek 8060 chemiluminescence nitrogen detector (Antek, Houston, USA). The column temperature was controlled by a Gynkotek column oven (Germering, Germany) at 250 °C.

**Reagents and buffers:** For preparation of the mobile phase, 0.5 mL of peptide-grade trifluoroacetic acid (TFA), (Solvay Fluor and Derivatives GmbH, Hannover, Germany) was added to each 1000 mL of water and methanol, gradient grade (Baker BV, Deventer, The Netherlands).

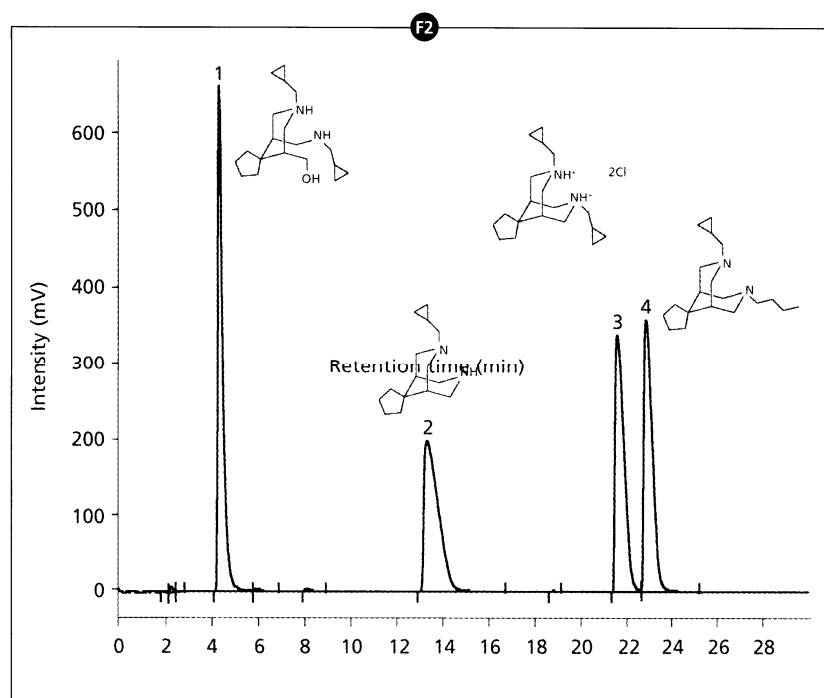
**Operating procedure, HPLC:** A flow-rate of 0.4 mL/min was adjusted, running isocratically 20% methanol/TFA for 5 min followed by a gradient to 90% methanol/TFA. Before entering the detector the flow was split to 0.25:0.15 mL/min using a T-piece and suitable PEEK capillaries to direct 0.15 mL/min to the input of the nebulizer.

**Operating procedure, CLND:** The detector was run at an oven temperature of 1050 °C with flow-rates of argon and oxygen adjusted to 180 and 135 mL/min, respectively. The nebulizer pressure was 20 psi. The ozone flow was adjusted to 25.6 and the high voltage of the PMT to -775 V. The PMT was chilled to -10 °C.

**Sample preparation:** Tedisamil dihydrochloride reference standard (0.5 mg/mL) was added to the buffered solution of the degradation product, which had been obtained by semipreparative liquid chromatography of



**Figure 1:** Chemical structure of tedisamil dihydrochloride and the degradation product 8857-19.



**Figure 2:** Chromatogram showing selectivity for tedisamil and three potential by-products. Peaks: 1 = 8857-10, 2 = 8857-9, 3 = Tedisamil, 4 = 8857-6.

the crude synthesis product. The solution was investigated by LC-electrochemical detection and LC-CLND in parallel.

**Method Development, Validation**

Analytical methods to be used within a Good Manufacturing Practice (GMP)-regulated environment must be validated according to requirements clearly defined in the corresponding ICH guideline (10). Apart from classic validation characteristics, proof of equimolarity is a new task, if one does not accept equimolarity in CLND as axiomatic, guaranteed by the design of the instrument and the chemical process. Indeed, to date, no deviations have been detected (11). However, because improper adjustments of the nebulizer may cause deviations, this item is more likely to be controlled during performance qualification or system suitability tests.

**Selectivity**

Selectivity was proven for a number of structurally related substances as is shown in Figure 2.

**Precision**

Precision was tested by injection of 50 µL of a solution containing approximately 25 mg/100 mL each of tedisamil dihydrochloride and three related substances 8857-8, 8857-9 and 8857-10, available as reference standards. Calculated for six injections, a coefficient of variance between 1.28 and 1.68% was determined. (The precision at a concentration of an impurity corresponding to 0.38% of the nominal concentration of tedisamil dihydrochloride was 4.5% determined from six runs within a sequence.)

**Linearity**

Linearity was confirmed within a range of 0.002–1 g/L of tedisamil dihydrochloride (i.e., 0.1–50 µg, corresponding to a range covering three orders of magnitude (Figure 3)). Solutions corresponding to each concentration level were injected once. The correlation coefficient was 0.99995.

**Equimolarity**

Equimolarity was tested by injecting a solution containing tedisamil dihydrochloride and the three potential synthesis by-products (cf., precision). The theoretical ratios of peak areas, calculated from the molecular weights of the hydrochlorides, are shown in Table 4.

**Limits of Detection and Quantification**

The limits of detection and quantification were determined by injecting a dilution of the solution used for determination of precision, with a final concentration of 5 µg/mL of each compound. The limits calculated from the signal-to-noise ratios according to the *European Pharmacopoeia* are listed in Table 5.

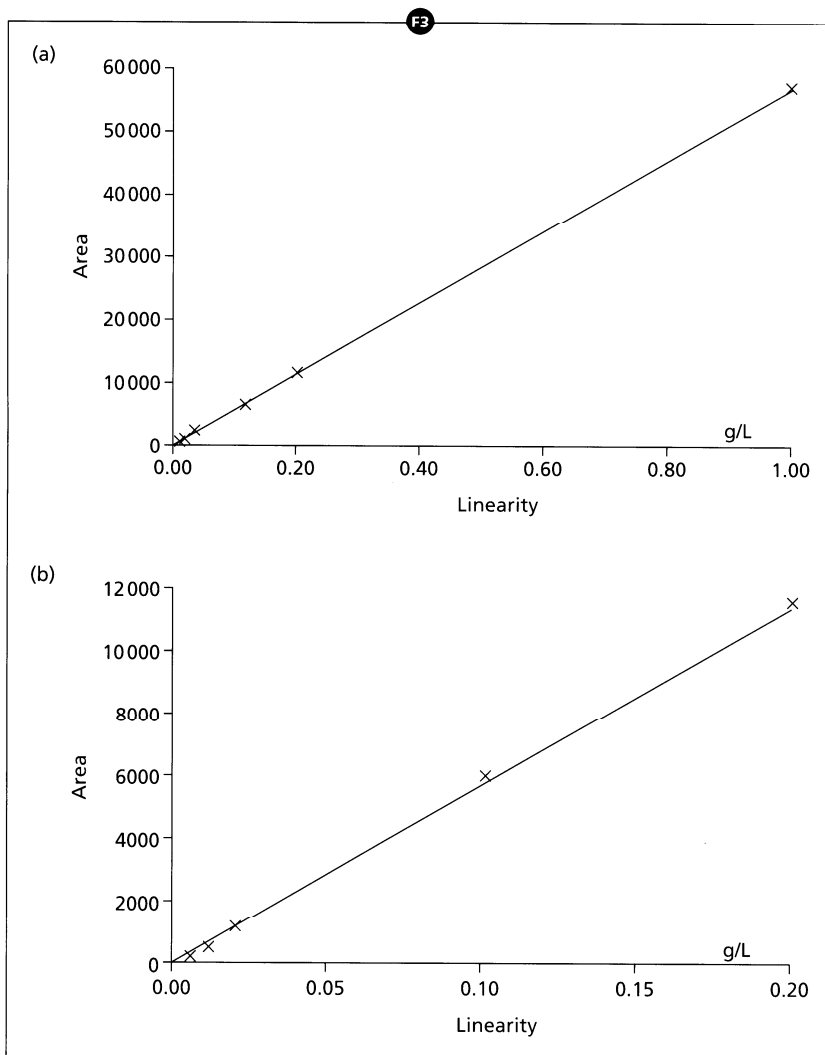
**Results**

The results are presented in Table 6 and the corresponding chromatograms are shown in Figure 4. A relative response factor of 1.3 was obtained, indicating that the peak areas from HPLC electrochemical detection must be corrected by this factor to obtain the exact amount of degradation product.

**Discussion**

Chemiluminescence nitrogen detection is a powerful tool for quantification of impurities in analytical development. It can replace classic procedures based on synthesis and characterization of reference standards, and probably erroneous approaches in which identical response factors for the main product and its related impurities are assumed. Method development is somewhat restricted because of special requirements for the coupling of HPLC to CLND. The drug substance and its impurities should be checked for suitability to this mode of detection taking into account the selectivity of the chromatographic system based on methanol/water with volatile buffers added and the nitrogen content of the compounds.

Performance of the method and apparatus was highly reliable, as demonstrated by the results of validation, including equimolarity. The method was shown to be suitable for impurity testing according to the requirements of the current ICH guidelines with predictable accuracy because of ruggedness of relative response factors. It was also shown useful to



**Figure 3:** Linearity of response versus concentration of tedisamil dihydrochloride in the concentration range from (a) 2–1000 mg/L and (b) 2–200 mg/L.

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**Table 4:** Peak Areas Obtained by CLND and Ratios Calculated versus Ratio Derived from Theory for Related Compounds.

Compound	MW	Area	Ratio (Calculated)	Ratio (Theory)
8857-9		8611004	0.97	0.96*
Tedisamil	361.4	8884168	1.00	1.00
8857-6	363.4	8976096	1.02	0.99
8857-10	379.4	8798459	0.99	0.95

\* Calculated from content of nitrogen obtained by elemental analysis.

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**Table 5:** Peak Areas and Ratios of Peak Areas for Tedisamil Dihydrochloride and the Degradation Product Obtained by Electrochemical Detection and CLND. The Relative Response Factor is 1.292.

Method	Peak Area*	Ratio
	-19	Tedisamil
ECD	408101	553548
CLND	656144	688955
		1.292

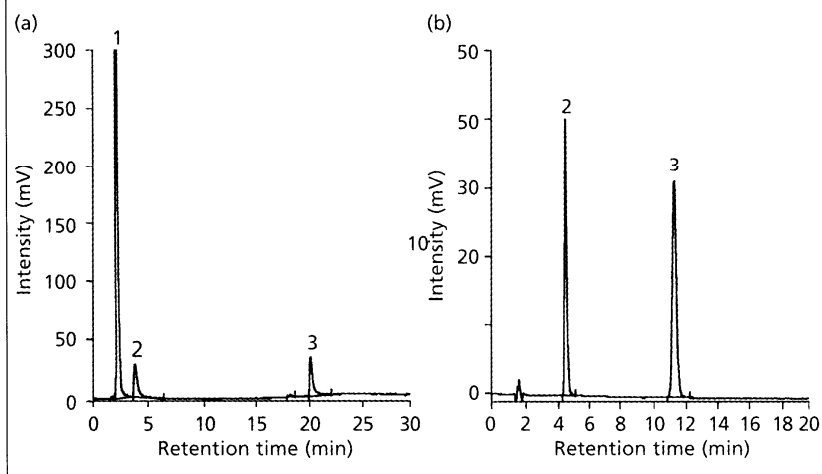
\* mean of six injections.

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**Table 6:** Limits of Detection (L.o.D.) and Limits of Quantification (L.o.Q.) Calculated for Tedisamil Dihydrochloride and Some Related Compounds.

	8857	8857-9	8857-10	8857-6
L.o.D. (µg/mL)	0.63	0.42	0.33	0.63
L.o.Q. (µg/mL)	2.09	1.39	1.10	2.11

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**Figure 4:** Chromatograms obtained by (a) CLND and (b) electrochemical detection after injection of a solution containing the degradation product and tedisamil as internal standard. Peaks: 1 = acetonitrile. 2 = 8857-19. 3 = tedisamil.

complete information on the accuracy of the routinely used method with electrochemical detection. Chemiluminescence nitrogen detection is considered a valuable addition to the strategies currently available to the pharmaceutical analyst, with regard to method validation and assaying of impurities.

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#### References

- (1) ICH Topic Q3B, Impurities in New Medicinal Products, Step 4, Consensus Guideline, 6 November 1996, The European Agency for Evaluation of Medicinal Products, Human Medicines Evaluation Unit.
- (2) ICH Harmonized Tripartite Guideline, Stability Testing of New Drug Substances and Products, Annex 1, Endorsed by the ICH Steering Committee at Step 4 of the ICH Process, 27 October 1993.
- (3) J. Duell et al., *J. Chromatogr.*, **392**, 175 (1987).
- (4) D.H. Fine and D.P. Roundbehr, *J. Chromatogr.*, **109**, 271-279 (1975).
- (5) E.M. Fujinari and J.D. Manes, *J. Chromatogr.*, **678**, 113-120 (1994).
- (6) R. Bizanek, J.D. Manes and E.M. Fujinari, *Peptide Res.*, **9**, 40-44 (1996).
- (7) H. Shi et al., *Chromatogr.*, **734**, 303-310 (1996).
- (8) J.T.B. Strode III et al., *Chrom. Sci.*, **36**, 511-515 (1998).
- (9) B.A. Olsen and M.D. Argentine, *J. Chromatogr. A*, **762**, 227-233 (1997).
- (10) ICH Topic Q2B, Validation of Analytical Procedures: Methodology, Step 4, Consensus Guideline, 6 November 1996, The European Agency for Evaluation of Medicinal Products, Human Medicines Evaluation Unit.
- (11) J.F. Borny, Antek 8060, Nitrogen Specific Detector, PowerPoint Presentation, Antek Inc., Houston, Texas, USA.

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