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Determination of neomycin and bacitracin in human or rabbit serum by HPLC–MS/MS

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Abstract

The method for the simultaneous determination of neomycin and bacitracin in human or rabbit serum was developed by using ion pairing reversed phase chromatography and tandem mass spectrometry (MS/MS) detection with electrospray (ESI) in positive mode. Both substances elute under these conditions at the same time and also kanamycin as internal standard elutes almost at the same time. The sample preparation was simple—only using 0.1 mL serum by protein precipitation with acetonitrile. Neomycin and bacitracin were detected as two-fold charged ions as well as the internal standard. The calibration range of these quite difficult detectable substances was 0.2–50 μg/mL of serum. The method was validated for both human or rabbit serum. The inter batch precision of quality control samples in human serum for neomycin ranged from 4.46% to 8.99% and for bacitracin from 6.85% to 11.17%. The inter batch accuracy for neomycin ranged from 98.7% to 100.7% and for bacitracin from 99.2% to 103.0%. At lower limit of quantitation (LLOQ) level of 0.2 μg/mL inter batch precision in human serum for neomycin was 12.05% and for bacitracin 11.91%, whereas accuracies were 99.9% for neomycin and 102.7% for bacitracin. Bench top stability in human or rabbit serum was given over three freeze thaw cycles and 4 h at room temperature.

The method can be considered to be specific and recoveries for sample preparation were high.

Keywords: Neomycin; Bacitracin; Liquid chromatography mass spectrometry; Human or rabbit serum; Antibiotics

1. Introduction

Determination of neomycin and bacitracin – both old antibiotics – is necessary because of checking systemic absorption for the two antibiotics which was not methodically feasible at that time when the antibiotics first entered into the market and law-requirements were not as strict as they are nowadays. Neomycin is ototoxic and nephrotoxic therefore oral absorption via intestinal tract or via damaged skin should be avoided. Bacitracin is nephrotoxic and therefore systemic absorption should be avoided. Even methods for determining only one of the two substances described in literature in biological samples are rare. There is no method described in literature for the simultaneous determination of both analytes together. For neomycin an aminoglycoside high-performance liquid chromatography (HPLC)–fluorescence after post column derivatization [1] with an LLOQ of 0.3 μg/mL of serum, TLC after derivatization with fluram [2] with a quantitation limit of 0.05 μg/mL of plasma or HPLC–tandem-MS [3] with a quantitation limit of 0.1 μg/mL, and an HILIC–tandem-MS method with a quantitation limit of 0.1 μg/mL serum [4] is described. For bacitracin only an ELISA [5] for the determination in biological samples and a review on identification of polypeptide antibiotics including bacitracin [6] are described. Both substances lack a usable chromophore but derivatization of the primary amino groups which both analytes possess is possible. As neomycin has six of them reproducibility of derivatization is quite a problem. On the other hand bacitracin as it is used for application consists of about nine different similar substances—all polypeptides. Simultaneous determination of both substances sometimes is necessary because of checking of oral or dermal application of both substances in one galenic formulation. The difficulty to determine bacitracin after derivatization (at least nine different polypeptides) and neomycin (six primary amino groups which cannot be derivatized equally precolumn, therefore post column derivatization only would work) should be avoided when using a HPLC–tandem-MS method. During method development the discovery was made that both analytes tend to strongly tail

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chromatographically and severe carryover was discovered for both analytes. Furthermore neomycin is hardly retained on a reversed phase column material. For both analytes ion yields in MS are low and a charge distribution occurs from one to multiple charges per molecule which decreases sensitivity, too. Furthermore charge distribution varies with analyte concentration which results in non-linear calibration curves for each charged ion. With ion pairing chromatography (with nonafluoropentanoic acid and formic acid) neomycin could be retained well on the chosen column as well as carryover could be eliminated and peak shape could be improved. Stabilisation of the charge could be gained from that (as well as by optimising the MS parameters) preferably to the doubly charged ion of the respective substance.

2. Experimental preface

2.1. Neomycin

In a first approach HPLC–MS in single ion monitoring (SIM) with atmospheric pressure chemical ionization (APCI) in positive ion mode was used for neomycin (Fig. 1, taken from Merck Index, 13th ed., 2001) and with ion pairing reagent enough retention was gained. Although different mobile phases were used in this phase with any of these injections of a dilution series (increments of factor of 10) of just the reference item resulted in non-linear response (30–60 times less signal for every 10th part of concentration). At that stage the distribution of charge was not checked and singly charged ions were monitored only. Of course similar results were obtained when monitoring in MRM mode. Similar results were gained by Heller et al. [7]. Since neomycin possesses six primary amino groups post column derivatization with NDA and OPA were checked. Both derivatization reagents resulted in adducts with neomycin which were not sensitive enough (about 5 ng amount detection limit). Later on charge distribution with HPLC–MS/MS for different multiply charged ions were checked and finally the decision was made to use the doubly charged ion and optimised all MS parameters.

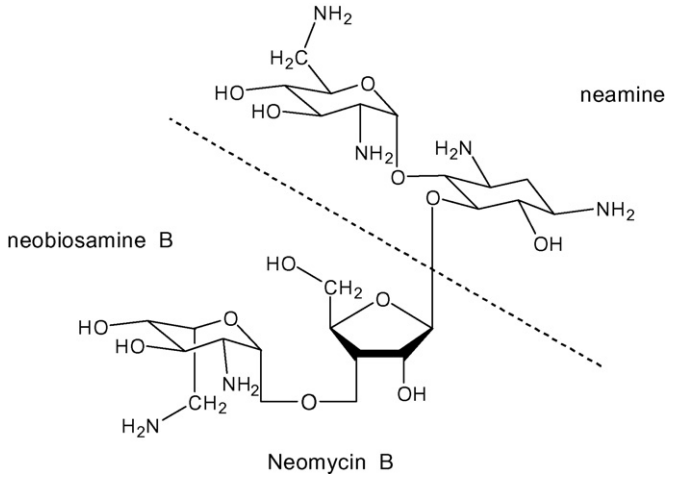

Fig. 1. Structure of Neomycin B.

2.2. Bacitracin

According to the Merck Index this molecule does not possess a definite molecular weight. Several components when first analysing this substance with HPLC–MS were indeed discovered (Fig. 2, taken from Merck Index, 13th ed., 2001). After post column derivatization with OPA there were two main peaks and at least nine components in total. Finally the decision was made to determine both neomycin and bacitracin simultaneously with HPLC–MS/MS with optimised ionization (doubly charged ions) and ion pairing chromatography in serum of humans and rabbits.

3. Experimental

3.1. Chemicals, reference items and matrices

Chemicals used were acetonitrile (gradient grade), formic acid (p.A.), and methanol (gradient grade) by Merck, Germany. Furthermore nonafluoropentanoic acid (NFPA, p.A.) provided by Sigma–Aldrich, USA; trifluoroacetic acid (TFA, purum) provided by Fluka, Switzerland, and water (ASTM-1 grade) by pharm-analyt, Austria.

Reference items were neomycin and bacitracin provided by Altana Pharma AG, Germany and the internal standard kanamycin provided by Sigma–Aldrich, USA.

Human blank serum used for spiking of calibration standards and quality control samples was pooled by Pharm-Analyt, Austria; rabbit blank serum used for spiking of quality control samples was provided by Kraeber, Germany.

Study samples derived from a dermal study with humans and from a rabbit parenteral study.

3.2. Sample preparation (human or rabbit serum)

After thawing at approximately 20–25 °C in a water bath or taking from ambient conditions (Standards, QC-samples), all samples used in a batch or analytical sequence were prepared as follows:

- One hundred microliters of the samples used were aliquot transferred into a test tube each. Thereafter 20 μL of IS work-

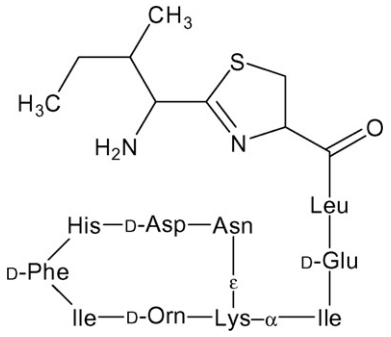

Fig. 2. Structure of bacitracin.