In Vitro Protein Binding of Liraglutide in Human Plasma Determined by Reiterated Stepwise Equilibrium Dialysis

ANNE PLUM,[1] LISBETH BJERRING JENSEN,[2] JESPER BØGGILD KRISTENSEN[3]

[1]Department of Diabetes Pharmacology PK/PD, Novo Nordisk A/S, Måløv DK-2760, Denmark

[2]Department of DMPK, Cell and Antibody Analysis, Novo Nordisk A/S, Måløv DK-2760, Denmark

[3]Chemistry and Isotope Laboratory, Novo Nordisk A/S, Måløv DK-2760, Denmark

Received 21 March 2013; revised 4 June 2013; accepted 10 June 2013

Published online 12 July 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.23648

ABSTRACT: Liraglutide is a human glucagon-like peptide-1 (GLP-1) analogue approved for the treatment of type 2 diabetes. It is based on human GLP-1 with the addition of a 16-carbon fatty acid, which facilitates binding to plasma proteins, thus prolonging the elimination half-life and allowing once-daily administration. It has not been possible to quantify liraglutide protein binding by ultrafiltration (the usual method of choice), as the lipophilic molecule becomes trapped in the filter membrane. Therefore, the aim of this study was to develop a methodology that could determine the extent of liraglutide binding to plasma proteins *in vitro*. We report here the details of a novel reiterated stepwise equilibrium dialysis assay that has successfully been used to quantify liraglutide plasma protein binding. The assay allowed quantification of liraglutide binding to proteins in purified plasma protein solutions and human plasma samples and was effective at plasma dilutions as low as 5%. At a clinically relevant liraglutide concentration (10^4 pM), greater than 98.9% of liraglutide was bound to protein. Specific binding to human serum albumin and α1-acid glycoprotein was 99.4% and 99.3%, respectively. The novel methodology described herein could have an application in the quantification of plasma protein binding of other highly lipophilic drug molecules. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:2882–2888, 2013

Keywords: liraglutide; type 2 diabetes mellitus; protein binding; plasma proteins; human serum albumin; *in vitro* method; equilibrium dialysis; insulin detemir; acylated peptides

INTRODUCTION

Glucagon-like peptide-1 (GLP-1) is an incretin hormone that induces glucose-dependent stimulation of insulin and reduction in glucagon secretion, delays gastric emptying and decreases appetite.[1,2] Consequently, there has been much interest in GLP-1 as a treatment for type 2 diabetes. However, its therapeutic application is limited by its rapid degradation by dipeptidyl peptidase-4 (DPP-4),[3] resulting in a half-life of approximately 1 h after subcutaneous administration.[4]

Liraglutide is a human GLP-1 analogue designed to provide a longer duration of action. The peptide is linked via a γ-L-glutamyl spacer to a 16-carbon fatty acid residue.[5] This facilitates reversible self-association and binding to serum albumin, slowing release from the injection site and reducing degradation by DPP-4, resulting in a plasma half-life of approximately 13 h.[6] Its apparent volume of distribution is low (11–17 L),[6] approximating the distribution volume of albumin, which is almost identical to the total volume of blood and interstitial fluid (~15 L),[7] thus indicating high levels of plasma protein binding.

Plasma protein binding of a drug can be altered by many factors, including disease state, age and concomitant therapies. Hepatic impairment and nephrotic syndrome, both of which are common among patients with type 2 diabetes and the elderly, are associated with hypoalbuminaemia,[8] which could potentially lead to altered protein binding levels of a drug. Plasma protein binding of a drug is clinically important, as it can affect the predictability of its pharmacokinetics, pharmacodynamics and dose–response relationship.[9] Plasma protein binding is critical to the protracted duration of action of liraglutide; therefore,

Correspondence to: Anne Plum (Telephone: +45-3075-4525; Fax: +45-4449-0555; E-mail: anp@novonordisk.com)

Journal of Pharmaceutical Sciences, Vol. 102, 2882–2888 (2013)

it is important to determine whether any of these conditions alter liraglutide plasma protein binding.

Standard methods for determining drug binding to plasma proteins include: *in vivo* measurements, gel filtration, ultracentrifugation, ultrafiltration and equilibrium dialysis. Ultrafiltration is the method of choice for drugs that are not quickly degraded or metabolised.[10] However, it has previously been observed that lipophilic drugs, such as liraglutide, insulin detemir and other drugs with 16-carbon fatty acid moieties, adsorb to the filter membrane rather than passing through it.[11] Initial attempts to quantify plasma protein binding using standard equilibrium dialysis were also hindered by adsorption to the dialysis membrane. To overcome this problem, we developed a hitherto unpublished reiterated stepwise equilibrium dialysis method, which has been utilised to measure the plasma protein binding of insulin detemir.[11]

This methodology was later optimised to quantify liraglutide plasma protein binding.[12] Here, we describe the reiterated stepwise equilibrium dialysis method, its validation and use to determine the extent of *in vitro* binding of liraglutide to plasma proteins.

MATERIALS AND METHODS

All studies were performed according to good laboratory practice, and all reports formed part of the documentation submitted to the health authorities for the approval of liraglutide.

Materials

Plasma was derived from ethylenediaminetetraacetic-acid-treated human blood, obtained under fasting conditions from two male and two female healthy volunteers who provided written informed consent. Liraglutide [molecular weight (MW) 3.751 kg/mol] was obtained from Novo Nordisk A/S (Bagsvaerd, Copenhagen, Denmark). Human serum albumin (HSA) and α1-acid glycoprotein (AAGP) were obtained from Sigma–Aldrich (Gillingham, UK).

Methods

Radiolabelling of Liraglutide and Stock Solution Preparation

Liraglutide was radiolabelled to allow quantification by liquid scintillation counting. [$^{125/127}$I Tyr 19]-labelled liraglutide was prepared and purified at the Chemistry and Isotope Laboratory, Novo Nordisk A/S, on the day before the dialysis incubation. Labelling was performed by the lactoperoxidase/hydrogen peroxide method, followed by purification and radiochemical purity analysis by high-pressure liquid chromatography as previously described.[13] The radiochemical stability of [$^{125/127}$I Tyr 19]-labelled liraglutide was improved by diluting the Na[^{125}I] 20-fold with Na[^{127}I] before iodination. This produced a tracer with low specific activity (0.1 μCi/pmol) and high radiochemical purity (>99%), which was maintained after 1 month at −20°C.

Liraglutide stock solution (10^7 pM) comprised 3.8 mg liraglutide and 40 μL iodinated liraglutide (corresponding to 10 μCi/mL) in 100 mL Krebs–Henseleit (KH) buffer (pH 7.4).[14] Different liraglutide concentrations were obtained (10^5 and 10^3 pM) by serial dilution, as appropriate. Final liraglutide concentrations of 10^6, 10^4 and 10^2 pM were obtained by 1:10 dilution into plasma. A plasma-free buffer incubation solution was also prepared.

Reiterated Stepwise Equilibrium Dialysis Assay

A standard equilibrium dialysis experimental set-up is illustrated. Figures 1a and 1b show the modified assay set-up, which was initiated with liraglutide in both chambers to avoid it becoming trapped in the dialysis membrane. Experiments were repeated at different across-membrane liraglutide concentration ratios (inner–outer) until liraglutide passage through the dialysis membrane was minimal, indicating that the system was at equilibrium.

The experimental set-up consisted of a Spectra/Por® Cellulose Ester Sterile DispoDialyzer sac (Spectrum Europe B.V., Breda, The Netherlands) containing 1.0 mL plasma (or HSA/AAGP) incubation solution with the appropriate concentration of liraglutide, dependent on the across-membrane concentration ratio required. Dialysis sacs had a diameter of 5 mm, a declared inner volume of 1 mL (although only an effective volume of 0.7 mL can be drawn) and a MW cut-off of 15 kDa, which provided the optimal balance between robustness and passage of the molecule. Dialysis sacs were mounted in an outer chamber filled with 12.5 mL buffer incubation solution, which contained the appropriate concentration of liraglutide in KH buffer. The outer chamber consisted of a screw-capped Minisorb tube (catalogue #366060; Nunc, Roskilde, Denmark), with an effective volume of 15 mL. The outer chamber was pre-coated with liraglutide by incubating twice with buffer incubation solution for 30 min at 37 ± 2°C in a Memmert thermostatic incubator (Memmert GmbH & Company KG, Büchenbach, Germany), followed by a final overnight incubation. Forty-five millilitre of buffer incubation solution (sufficient buffer for triplicate incubations) was prepared in 50 mL screw-capped Minisorb tubes (PBC catalogue #339497; Nunc).

Following pre-coating, dialysis sacs were mounted into the outer buffer chambers and incubated at 37 ± 2°C in a Memmert thermostatic incubator for 4–5 h