FlashPlate® File #8

A Simple High Throughput FlashPlate Assay to Quantify ET-Antagonists in Plasma

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Abstract

Described is an assay combining liquid extraction and radioligand competition binding suitable for the quantification of endothelin (ET) antagonists in plasma, urine and tissue. The results of this assay, as obtained using conventional methods, are compared to those obtained using the FlashPlate platform from PerkinElmer Life Sciences.

The assay system allows quantitative analysis of bosentan (a recently described potent, orally active ET antagonist on ET_A and ET_B receptors) in plasma of different species, including man, rat, rabbit, dog and marmoset. A lower detection limit of 20 ng/ml was reached using as little as 50 µl plasma, allowing pharmacokinetic studies in rats. The assay principle does not differentiate between parent compound and circulating active metabolites. Thus, by comparison with HPLC data, it is possible to estimate the contribution of active metabolites to activity. No circulating active metabolites were detected for bosentan using this methodology.

The assay was further developed on the FlashPlate platform, eliminating the extraction procedure, and resulting in an overall assay time of less than four hours, compared to approximately 24 hours using conventional extraction methodology. Using this improved method, the ET-receptor antagonists bosentan and SB 209670 could be quantified in rat plasma with detection limits of 15 and 4.5 ng/ml, respectively.

Background and Objective

Reliable pharmacokinetic data is essential for final selection of endothelin (ET) antagonists to be considered for clinical development. Thus, an accurate, sensitive, and fast method to assess plasma levels after both parenteral and oral application is needed. Small amounts of plasma should be sufficient for this assay to allow repeated sampling in small animals, such as rats.

The aim of this study is the development of a radioligand competition binding assay suitable for assessment of plasma levels of bosentan and other ET-antagonists.[1,2]

Methods

Extraction Procedures for Endothelin and Bosentan

As a general extraction method for ET, 1 ml methanol is added to 50 µl plasma, urine or tissue extract. After vortexing (10 sec.) and additional shaking (10 min.), precipitated proteins are removed by centrifugation (3,000 x g, 30 min.), the supernatant is evaporated to dryness, re-dissolved in 200 µl of 75 mM Tris buffer (pH 7.4, 25 mM $MnCl_2$, 1 mM EDTA 0.5% BSA), and transferred to 96-well microplates.

The above procedure was followed to extract Bosentan (Ro 47-0203) from plasma, with the following exceptions: centrifugation was performed for 5 minutes (at 3,000 x g); and after evaporation of the methanol phase, samples were redissolved in 50 mM Tris buffer.

Conventional Binding Assay Protocol

This method is modified from Breu, *et al.*[3] Baculovirus-infected insect cells, expressing either recombinant ET_A or recombinant ET_B receptor cloned from human placenta, were broken by three freeze/thawing cycles in hypotonic Tris buffer (5 mM, pH 7.4, 1 mM $MgCl_2$), then homogenized, and centrifuged at 72,000 x g for 15 min. The pellet was washed twice with 75 mM Tris buffer (pH 7.4, containing 25 mM $MgCl_2$ and 250 mM sucrose), resuspended in the same buffer, and stored in aliquots at -80˚C. Protein was determined according to Lowry's method using BSA as a standard.

Competition binding assays were performed on membrane preparations or baculovirus-infected insect cells expressing recombinant human ET_A receptor. Binding assays on membranes were performed in 250 µl 50 mM Tris buffer (pH 7.4, 25 mM $MnCl_2$, 1 mM EDTA, 0.5% (w/v) BSA) containing 5 µg protein, 32 pM [^{125}I]-ET and increasing amounts of unlabeled ligands. Incubation was performed for three hours at room temperature. Separation of bound and free ligand was achieved by filtration. Each assay was performed three times in triplicate and non-specific binding (NSB) was assessed in the presence of 100 nM unlabeled ET-1.

Specific binding was defined as the difference between total binding and non-specific binding. IC_{50} values were determined after logit/log transformation of the binding data.

Concentrations of bosentan were computed from a calibration curve in spiked plasma. The lower sensitivity limit was 20 ng/ml. Concentrations above the upper limit (2 µg/ml) were measured after appropriate dilution with Tris buffer. All measurements were performed as triplicate determinations.

Binding Assay Using FlashPlate Microplate

FlashPlate (SMP200) is a 96-well polystyrene microplate with plastic scintillator coated wells. It is designed for use with the TopCount® Microplate Scintillation and Luminescence Counter (Packard Instrument Company) and similar instruments.

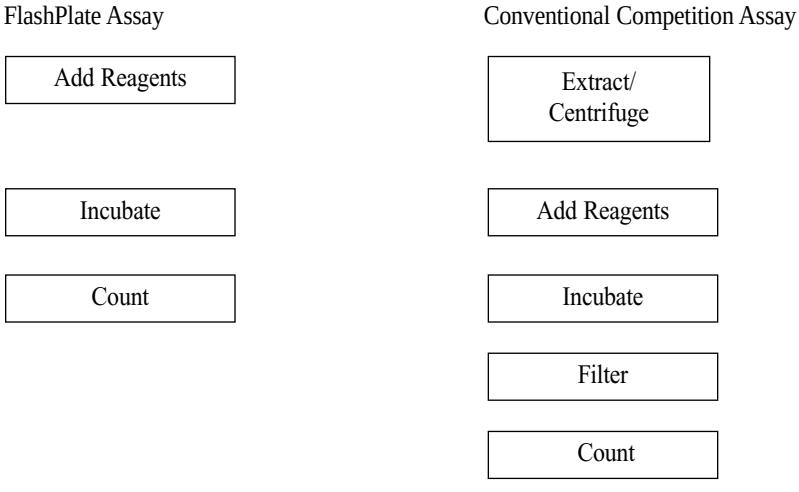
The FlashPlate-based assay method follows:

- Precoat with polyethylene imine (0.1%) in phosphate buffer (0.2 M Na_2HPO_4/citrate; pH 7.4) for 24 hours, 4˚C.
- Wash (3x) with phosphate buffer.
- Coat with recombinant ET_A receptor (5 µg protein/well) in 100 µl phosphate buffer for 16-24 hours.
- Block nonspecific binding sites by treatment with 1% BSA (1 hour, room temperature). In this state, plates can be stored for six weeks without significant loss of binding capacity.
- Binding assay: add 75 µl of Tris buffer (50 mM Tris, 1 mM EDTA, 0.01% NaN_3, 0.5% BSA, 25 mM $MnCl_2$, pH 7.4), 50 µl plasma and 125 µl Tris buffer with [^{125}I]-ET-1 (30,000 cpm).
- Incubate for three hours at room temperature. No filtration or centrifugation steps are required.
- Count on a Packard TopCount.

Using the FlashPlate-based technique, analysis of plasma samples with unknown concentration can be completed in less than four hours of assay time. In comparison, use of the conventional assay methodology requires approximately 24 hours. See Figure 1.

Figure 1

Protocol Comparison



Results

FlashPlate-based assays do not require separation of free and bound radioligand. The incubation is not interrupted prior to measurement. Thus, the FlashPlate platform is especially well suited for efficient kinetic studies. See Figures 2 and 3.

Figure 2

Competition binding curve of ET-1, measured on FlashPlate

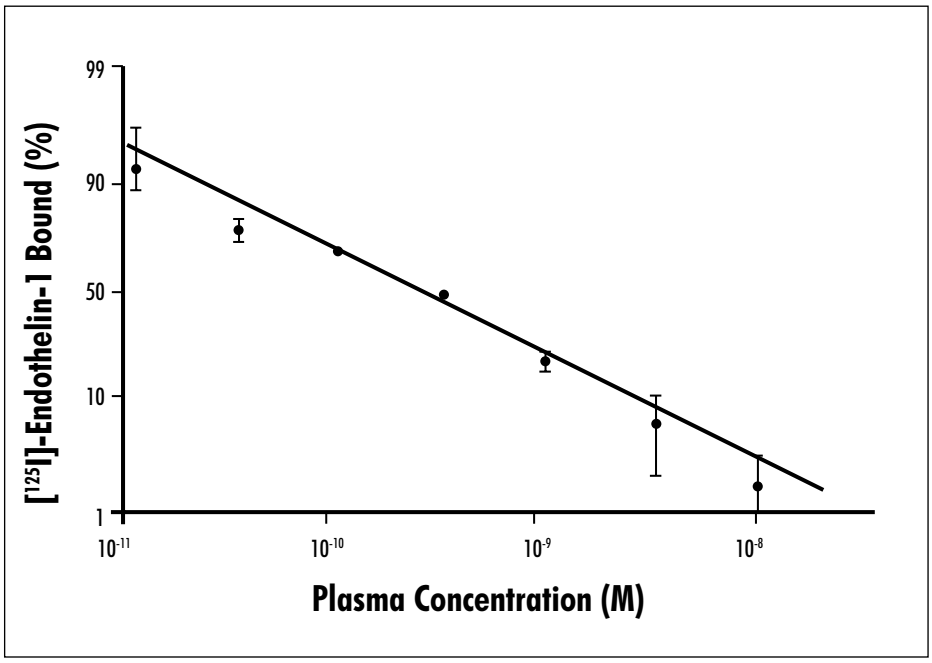
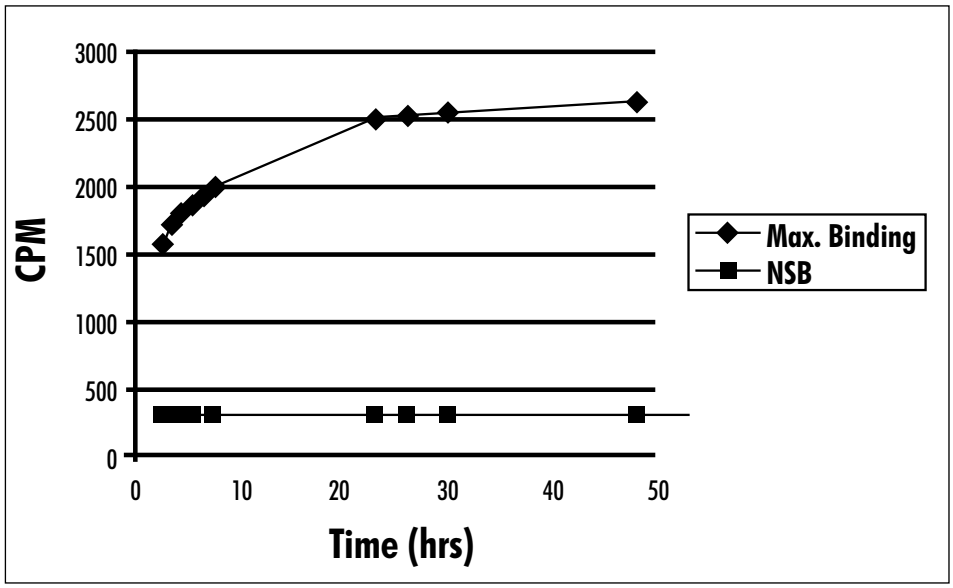


Figure 3

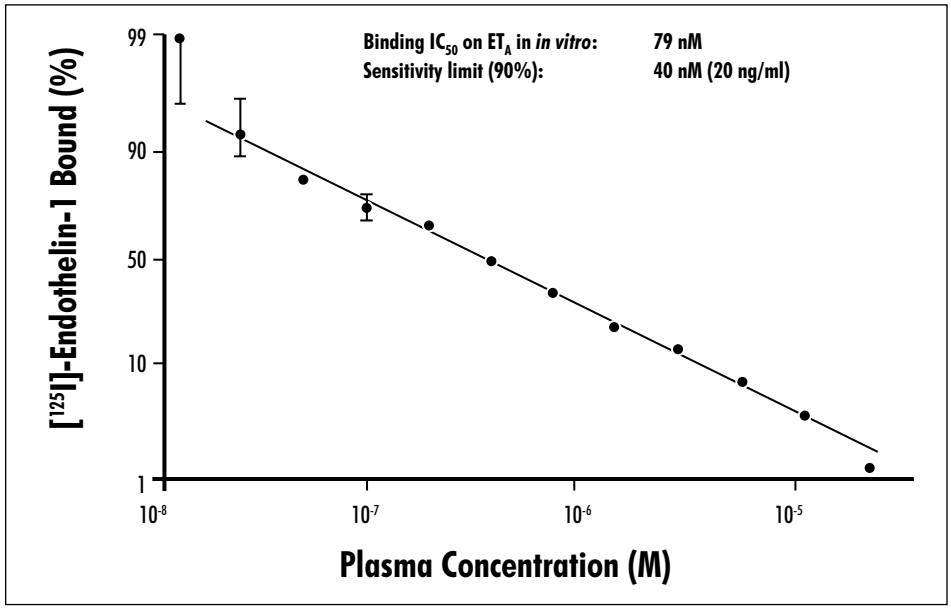
Repeated measurements of the same FlashPlate, providing a time course for the binding of [^{125}I]-ET-1.



The conventional assay is able to detect both parent compound and active metabolites. It is thus possible to estimate the contribution of active metabolites to activity by comparison with HPLC data. For bosentan, no major circulating active metabolite was found. See Figure 4.

Figure 4

Calibration curve for the quantification of bosentan in human plasma, after extraction as performed by conventional binding assay.



Using FlashPlate, bosentan and SB 209670 can be quantified in plasma without time-consuming extraction. See Figures 5 and 6.

Figure 5

Calibration curve for the quantification of bosentan in human plasma: assay performed in FlashPlate.

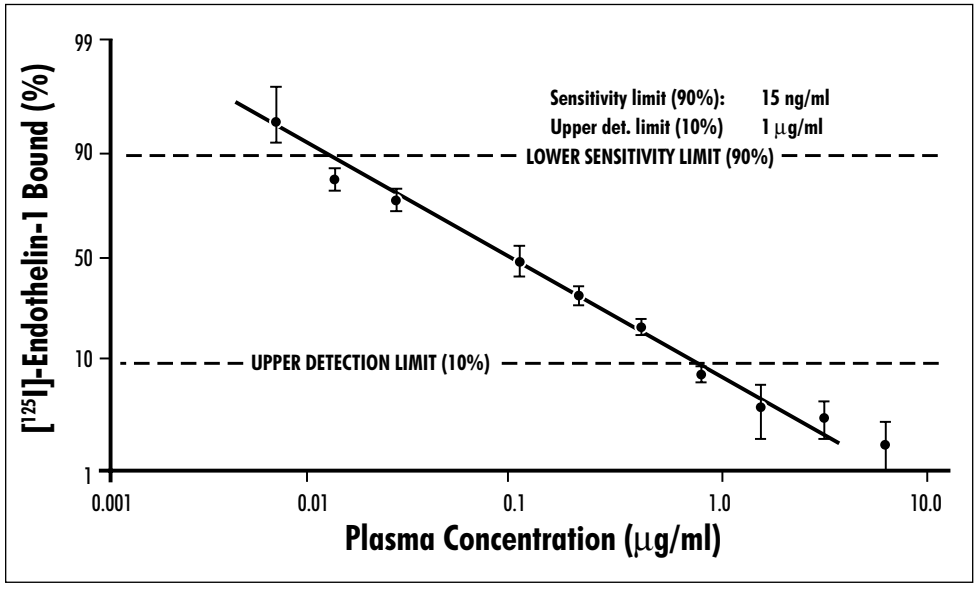
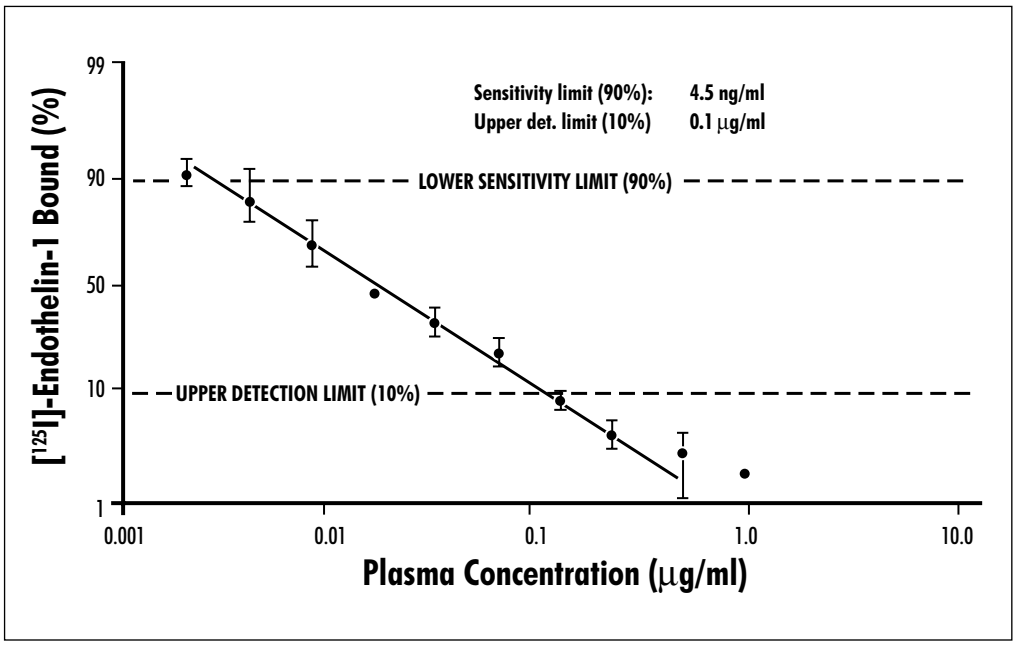


Figure 6

Calibration curve for the quantification of SB 209670 in rat plasma: assay performed in FlashPlate.



Summary

The combination of simple liquid extraction with a radioligand competition binding assay provides a high throughput method for the quantification of endothelin antagonists in plasma.

This assay exhibits several advantages:

- It is well suited to 96-well microplates.
- It provides high sensitivity (detection limit: 20 ng/ml for bosentan).
- Only a small volume of plasma is required (< 50 µl).
- It additionally detects active metabolites.
- It can be easily modified to measure:
  a) plasma from different species.
  b) urine and cerebrospinal fluid.
  c) structurally different ET-antagonists, e.g., SB 209670 (detection limit: 4.5 ng/ml).

The assay procedure is simplified and optimized by using FlashPlate technology to avoid the time-consuming extraction procedure. This method easily adapts to automation if large numbers of samples are to be processed, yielding significant time and labor savings.

References

1. Clozel, M., V. Breu, K. Burri, J.-M. Cassal, W. Fischli, G.A. Gray, G. Hirth, B.-M. Löffler, M. Müller, W. Neidhart, and H. Ramuz. 1993. Pathophysiological role of endothelin revealed by the first orally active endothelin receptor antagonist. *Nature* 365:759-761.
2. Clozel, M., V. Breu, G.A. Gray, B. Kalina, B.-M. Löffler, K. Burri, J.-M. Cassal, M. Müller, W. Neidhart, and H. Ramuz. 1994. Pharmacological characterization of bosentan, a new potent orally active nonpeptide endothelin receptor antagonist. *J. Pharmacol. Exp. Ther.* 270:228-235.
3. Breu, V., M. Clozel, and B.-M. Löffler. 1993. In vitro characterization of Ro 46-2005, a novel synthetic non-peptide endothelin antagonist of ET_A and ET_B receptors. *FEBS Letters* 334: 210-214.

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