Quantification of ligand PET studies using a reference region with a displaceable fraction: application to occupancy studies with [^{11}C]-DASB as an example

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This paper aims to build novel methodology for the use of a reference region with specific binding for the quantification of brain studies with radioligands and positron emission tomography (PET). In particular: (1) we introduce a definition of binding potential $BP_D = DVR - 1$ where DVR is the volume of distribution relative to a reference tissue that contains ligand in specifically bound form, (2) we validate a numerical methodology, rank-shaping regularization of exponential spectral analysis (RS-ESA), for the calculation of BP_D that can cope with a reference region with specific bound ligand, (3) we demonstrate the use of RS-ESA for the accurate estimation of drug occupancies with the use of correction factors to account for the specific binding in the reference. [^{11}C]-DASB with cerebellum as a reference was chosen as an example to validate the methodology. Two data sets were used; four normal subjects scanned after infusion of citalopram or placebo and further six test–retest data sets. In the drug occupancy study, the use of RS-ESA with cerebellar input plus corrections produced estimates of occupancy very close the ones obtained with plasma input. Test–retest results demonstrated a tight linear relationship between BP_D calculated either with plasma or with a reference input and high reproducibility.

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Introduction

Background

Radioligand imaging allows the quantification of neuroreceptor availability and drug occupancies providing *in vivo* data on the neurobiology of diseases and drug treatments. Where there is a brain region that does not show specific binding to the radioligand, this region can be used as a reference region, obviating the need for a plasma input

function. This greatly simplifies the practical application of the procedure, and makes studies in patient groups who may not tolerate arterial blood sampling possible. However, a brain region that does not show specific binding does not exist for many radioligands. An approach that allows the use of a reference region that shows some specific binding would simplify the practical application of these radioligands. This paper addresses this issue by proposing and validating an approach that allows the quantification of neuroreceptor availability using a reference region that shows specific radioligand binding.

The mathematical procedures for the quantification of radioligand dynamic studies measured with positron emission tomography (PET) are generally based on compartmental models, where exchanges among body compartments are described by a system of first order differential equations (Schmidt and Turkheimer, 2002). Analytical solution of such a

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system produces an equation of the general form (Gunn *et al*, 2001):

$$c(t) = \sum_{i=1}^N \alpha_i e^{-\beta_i t} \otimes u(t) + \alpha_0 u_0(t) \quad (1)$$

$$\beta_i \geq 0,$$

In equation (1) $c(t)$ is the measured tissue radioactivity, $u(t)$ is the input function that can be obtained from arterial plasma or from a reference region and $\otimes$ is the convolution operator. In the plasma input case, where $u(t)$ represents the plasma parent input function, $u_0(t)$ is whole blood radioactivity in the tissue. In the special case of a reference region input, $u_0(t) = u(t)$. In the model in equation (1) α_i is unconstrained and the model order N is unknown. All time courses in this equation are assumed to be precorrected for radioactive decay.

For plasma input models, estimation of equation (1) allows the calculation for a target region of the total volume of distribution V_T defined as:

$$V_T = \sum_{i=1}^N \alpha_i / \beta_i \quad (2)$$

For reference tissue models, estimation of equation (1) leads to the direct calculation of the relative volume of distribution (DVR):

$$DVR = \sum_{i=1}^N \alpha_i / \beta_i + \alpha_0 \quad (3)$$

Where:

$$DVR = \frac{V_T}{V_{REF}} \quad (4)$$

V_{REF} is the volume of distribution of the reference tissue. When the reference region contains the radiotracer in nondisplaceable, i.e., free and nonspecifically bound, states only, V_{REF} can be termed V_{ND} .

The presence of a reference tissue with no displaceable component allows the calculation of the binding potential BP_{ND} as:

$$BP_{ND} = \frac{V_T - V_{ND}}{V_{ND}} = \frac{V_T}{V_{ND}} - 1 = DVR - 1 \quad (5)$$

The interested reader is referred to Innis *et al* (2007) for nomenclature and complete definitions of volumes of distributions and binding potentials.

It is often the case that a reference region in the brain does not exist. It is, therefore, of interest to develop quantitative and numerical methodology that uses as input function the time-activity curve (TAC) of a region that contains the specific target of

the radioligand albeit in low-to-moderate quantity. Therefore, this paper aims to:

1. Define BP_D as the binding potential obtained using a reference with displaceable binding.
2. Discuss the potential advantages and pitfalls of BP_D as a quantitative tool in dynamic PET studies.
3. Validate a numerical methodology of general use for the calculation of BP_D , able to cope with any compartmental structure for the reference region.
4. Illustrate the use of BP_D to obtain accurate estimates of the occupancy in the case of competition studies by using predefined correction factors based on *a-priori* information on the volume specifically bound in the reference.

We then illustrate the methodology using [^{11}C]-DASB data sets with and without plasma input function as well as test-retest data for the same tracer to ascertain the stability of the numerical estimates. Throughout the manuscript, compartmental model with plasma or reference input is used as a reference.

Materials and methods

Theory

Reference with displaceable fraction: In more general terms, a binding potential BP refers to specific binding concentration as a ratio to other concentrations (i.e., total in plasma, free in plasma, and free plus nondisplaceable in case of BP_{ND} ; Innis *et al*, 2007). We can, therefore, introduce a definition of BP where specific binding is referred to a more general reference tissue where the radioligand is present in free, nondisplaceable, and displaceable form:

$$BP_D = \frac{V_T - V_{REF}}{V_{REF}} = \frac{V_T}{V_{REF}} - 1 = DVR - 1 \quad (6)$$

The use of BP_D is more problematic than BP_{ND} . In the case of BP_{ND} , the nondisplaceable fraction depends largely on the binding of the ligand to the cellular lipid bilayer (Rosso *et al*, 2008), which can be usually assumed constant throughout an organ of interest (e.g., brain). The non-specific binding fraction is also less likely to be altered in disease states and should not change in occupancy studies with pharmacological doses of an unlabelled drug.

BP_D instead depends on the size of the displaceable volume of the reference region. This volume, which is related to the density and affinity of the particular receptor system in that anatomical location, will vary depending on the region selected as reference. Because it contains a certain concentration of the binding sites of interest, such a reference region is likely to be altered by disease states and is definitively altered by pharmacological blocking agents specific for those sites. With the caveats above, there are, however, at least two instances where BP_D can have a use.

The first instance is of a radiotracer where displaceable fractions exist throughout the organ of interest, but there is

evidence of at least one regional-specific concentration that is left unaltered in the pathology under study. It is reasonable to assume that such evidence may be obtained, for example, by pathological examination of postmortem tissue as in the work of Gulesserian *et al* (2000) that demonstrated no change in serotonin transporter (SERT) concentration in the cerebellum of Alzheimer's disease patients.

The second instance is the case of drug occupancy PET studies using a radiotracer that has displaceable fractions across the whole organ of interest, but with a region where the displaceable fraction is small and, more importantly, known up to a certain accuracy. As an example that will be considered further in this manuscript, we consider a bioassay that measures the occupancy of a hypothetical selective serotonin reuptake inhibitor on the SERT using PET and [^{11}C]-DASB.

[^{11}C]-DASB as an example: [^{11}C]-DASB is a highly selective ligand for SERT, not only with nanomolar affinity for the SERT but also with micromolar affinity for the dopamine and norepinephrine transporters (Houle *et al*, 2000). In cerebellum, the reference region of choice for this tracer, a displaceable fraction of up to 33% has been demonstrated after acute sertraline treatment (Parsey *et al*, 2006). The presence of specific binding in the reference region should in principle discourage the use of a reference modelling approach in a [^{11}C]-DASB PET bioassay or, in other words, for pharmacological studies for novel selective serotonin reuptake inhibitors. Figure 1 illustrates the bias resulting from the estimation of drug occupancy for [^{11}C]-DASB assuming a typical range of specific volumes in

the reference. In the figure, the x axis reports the 'true' occupancies, while occupancies $Occ(y)$ on the y axis were calculated as:

$$Occ(y) = \frac{DVR^b - DVR^o}{DVR^b - 1} \quad (7)$$

DVR^b and DVR^o are, respectively, the relative volume of distribution at baseline and after exogenous displacement and are defined as:

$$DVR^b = \frac{V_{ND} + V_S}{V_{ND} + V_S^R} \quad (8)$$

$$DVR^o = \frac{V_{ND} + (1 - Occ) \times V_S}{V_{ND} + (1 - Occ) \times V_S^R} \quad (9)$$

V_S and V_S^R are the displaceable volume in the target and in the reference, respectively. It is useful to express V_S^R as percentage of the nondisplaceable volume V_{ND} , that is:

$$V_S^R = p \times V_{ND} \quad (10)$$

By inserting equation (10) in equations (8, 9) and the resulting definitions of DVR^b and DVR^o in equation (7), after simple rearrangements the dependency of $Occ(y)$ from V_{ND} , V_S , and V_S^R vanishes and one obtains:

$$Occ(y) = 1 - \frac{(1 + p) \times (1 - Occ)}{(1 + p - p \times Occ)} \quad (11)$$

Note that equation (11) is independent from the absolute values of the volumes of distribution of the target and reference region and depends solely on the displaceable fraction in the reference p .

Figure 1 then illustrates the bias profiles for instances of the displaceable volume in the reference ranging from 0% to 50% (note that the percentage of total reference volume is equal to $p/(1 + p)$).

In general terms, one notes that the bias introduced in the measured occupancy by using a reference region for quantification is small. If the value of the displaceable volume in the reference region is known, the bias can be readily obtained from equation (11) and the measured occupancy can be corrected accordingly. Importantly, the correction may be effective even if the volume in the reference is not known with great precision. In Figure 1, in the case of a drug induced 50% true occupancy, reference modelling would produce an estimated erroneous occupancy, respectively, of 38% or 42% depending on whether the specific volume in the reference is 10% or 30% of the total. In other words, a $\pm 20\%$ difference in the assumed displaceable volume translates to a 4% difference in the estimated bias which is then reflected in the occupancy estimate.

Accurate estimation of nondisplaceable binding: The previous paragraph indicated that occupancy studies using a reference region with a displaceable volume require *a-priori* knowledge of the displaceable volume in the reference. This is achievable with a preliminary PET study with a pharmacological challenge by a known specific

Figure 1 Illustration of the bias in the calculation of drug occupancy induced by the use of the cerebellum as a reference using typical [^{11}C]-DASB data. The biased occupancy on the y axis is $Occ(y) = 1 - ((1 + p) \times (1 - Occ)) / (1 + p - p \times Occ)$ where p is displaceable volume as percentage of the nondisplaceable in the reference. Bias is simulated for five increasing values of the displaceable fraction (in percentage equal to $p/(1 + p)$, range 10% to 50%) of the total cerebellar volume.

blocker with the further requirement of a plasma input function. In other words, the PET-radiotracer bioassay has to be fully characterized before being used for further pharmacological investigations using novel compounds. For radiotracers, where a 'true' reference region does not exist, estimates of the nondisplaceable volume can be obtained by a two-scan procedure, with the second scan using a coinjected cold drug amount. By using a plasma input function to calculate the regional total volumes of distributions V_T^1 and V_T^2 , respectively, for the baseline scan and the scan after administration of the cold drug, one can write (Cunningham *et al*, 2010; Lassen *et al*, 1995):

$$(V_T^1 - V_T^2) = Occ \times V_T^1 - Occ \times V_{ND} \quad (12)$$

Equation (12) requires minimal assumptions (e.g., non-specific binding has to be homogeneous in brain and occupancy is the same in all regions) and allows estimation of Occ and V_{ND} through linear regression of $(V_T^1 - V_T^2)$ versus V_T^1 .

V_{ND} and the volume of distribution V_T^1 of the reference region of choice can then be used to estimate the bias correction in equation (11) for further use in blocking studies without blood sampling.

Quantification using a displaceable reference: The theory developed so far is based on the important caveat that a computational procedure exists that can compute DVR from PET dynamic data using the TAC of a reference tissue as input function irrespective of its compartmental structure. Popular reference tissue modelling approaches such as the simplified reference tissue model (SRTM; Gunn *et al*, 1997; Lammertsma and Hume, 1996) and the reference tissue Logan plot (Logan *et al*, 1996) are based on the assumption of a mono-compartmental structure of the reference region. In the context of this work, the reference region will possibly have a multicompartmental structure because of the displaceable fraction; and therefore, the above-mentioned methods may not be suitable. For this reason, in this work we used the rank-shaping regularization of exponential spectral analysis (RS-ESA; Turkheimer *et al*, 2003), which is a development of the original Exponential Spectral Analysis (ESA; Cunningham and Jones, 1993; Turkheimer *et al*, 1994) applicable to reference tissue modelling. ESA adopted a basis-function approach for the solution of equation (1) through the definition of a very large set of convolution integrals for a fixed set of β_i and the estimation process is resolved by the nonnegative least squares algorithm that produces a sparse solution with a very small number of nonzero and positive α_i . Therefore, ESA does not need a defined compartmental structure. However, because of the positivity constraints, it cannot be used for reference region modelling (Turkheimer *et al*, 2003). RS-ESA works on the same principles but, differently from ESA, it does not rely on the nonnegativity constraints of nonnegative least squares and can, therefore, be used with a reference input (Turkheimer *et al*, 2003, 2007).

Subjects and Medication

For the occupancy study, the data already published in Hinz *et al* (2008) were reanalyzed. In short, four healthy

male volunteers (aged 37, 42, 49, and 57 years) were recruited and underwent two PET scans with [^{11}C]-DASB after either placebo or citalopram infusion. Subjects were studied at least a week apart for both experiments, mean interval was 9 ± 3.4 days. The intravenous infusion of either 10 mg of citalopram or placebo (saline) was administered over at least half an hour starting 45 minutes before injection of the radioligand. The order of citalopram or placebo was randomized. The dose and timing of the citalopram infusion were determined on observations documented in previous studies where the effects of citalopram on anterior pituitary hormones, which are established markers of serotonergic system integrity, were reported (Attenburrow *et al*, 2001; Bhagwagar *et al*, 2002).

For the test-retest study, six healthy male volunteers (aged 35, 37, 58, 52, 45, and 57 years) were recruited and scanned twice with a week interval.

Psychiatric screening (including substance/alcohol use history) and physical assessment were carried out by a qualified clinician. None of the subjects met Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria for current or past depressive or anxiety disorders, and all were physically healthy. In addition, confirmation about the health status of all the consenting volunteers was obtained from their general practitioners.

The Research Ethics Committee of the Hammersmith Hospitals Trust and the ARSAC (Administration of Radioactive Substances Advisory Committee) of the United Kingdom approved the study. International Conference on Harmonization-Good Clinical Practice (ICH-GCP) guidelines were followed in designing, running, and in the analysis process.

Radiochemistry

[^{11}C]-DASB was synthesized as described earlier through the reaction of [^{11}C]methyl iodide with the desmethyl precursor (Wilson *et al*, 2002). The precursor desmethyl DASB was supplied by the Target Molecules Ltd. (Southampton, UK). [^{11}C]-DASB was administered via injection into an antecubital vein as a smooth bolus over 30 seconds. The injected radioactivity was between 487 and 547 MBq (mean: 523 MBq, SD: 24 MBq) for the occupancy study and it was between 531.70 and 567.77 MBq (mean: 541.27 MBq, SD: 17.08 MBq) for the test-retest study. The radiochemical purity of the injected [^{11}C]-DASB was high and ranged from 95% to 100% (mean: 97.58%, SD: 1.4%).

Data Acquisition

The PET data were acquired on a high-sensitivity ECAT EXACT3D (Siemens/CTI, Knoxville, TN, USA) scanner with an axial field of view of 23.4 cm and 95 reconstructed transaxial image planes (Spinks *et al*, 2000). A 5-minute transmission scan with a [^{137}Cs] rotating point source was performed before each emission scan for scatter and attenuation correction (Watson *et al*, 1996). An annular side shielding was used to reduce the counts deriving from activity outside the direct field of view (Spinks *et al*, 1998). The 90-minute three-dimensional dynamic emission scan

was acquired in list mode. In the postacquisition frame rebinning, 28 time frames of increasing length were generated (30 seconds background frame, one 15 seconds frame, one 5 seconds frame, one 10 seconds frame, three 30 seconds frames, three 60 seconds frames, three 120 seconds frames, three 180 seconds frames, eight 300 seconds frames, and four 450 seconds frames). The spatial resolution of the images reconstructed using the reprojection algorithm with the ramp and Colsher filters set to Nyquist frequency was 5.1 mm full width at half maximum transaxially and 5.9 mm full width at half maximum axially averaged over a radius of 10 cm from the center of the field of view (Spinks *et al*, 2000).

Magnetic Resonance Imaging

For each subject, magnetic resonance imaging (MRI) data were acquired to have an anatomical reference. It was not possible to acquire all the MR imaging data on the same scanner because of technical problems and decommissioning of scanners during the study. Consequently, the MRI was performed on scanners with a field strength of either 0.5 T (0.5 Apollo System, Marconi Medical Systems, Cleveland, OH, USA) or 1.5 T (1.5 Eclipse System, Marconi Medical Systems) (repetition time = 30 ms, echo time = 3 ms, flip angle = 30°, number of acquisitions = 1, voxel dimensions 0.98 × 1.6 × 1.6 mm, acquisition time = 13 minutes). The scans were all examined by an independent clinical neuroradiologist for abnormalities. No participants were excluded on the basis of this examination.

Image Analysis

Head movement in the dynamic PET acquisition was corrected for using frame-by-frame realignment. Non-attenuation corrected images were used to optimize the frame-by-frame realignment process (Montgomery *et al*, 2006); the frame with the higher count rate was used as reference. Dynamic scans were denoised using a level 2, order 64 Battle Lemarie wavelet filter (Turkheimer *et al*, 1999). A mutual information algorithm (Studholme *et al*, 1997) was applied for frame realignment to a single frame in which there was a high signal-to-noise ratio based on the global count-rate data obtained from the scanner. Transformation parameters were applied to the corresponding attenuation-corrected dynamic images to generate a movement-corrected dynamic image.

MRI images were first segmented in gray/white matter/cerebrospinal using the segmentation tool in SPM5 (<http://www.fil.ion.ucl.ac.uk/spm>). To match anatomical and functional information, MRI images were then resliced (1 × 1 × 1 mm³) and coregistered to the corresponding subject's summed PET image (activity summed along the interval 9 to 90 minutes) using the normalized mutual information method which implements a rigid body transformation in SPM5.

The selection of the regions of interest (ROIs) was based on the distribution of SERT and seven regions in total were included in the investigation. More specifically, SERT moderate to rich regions like brainstem, thalamus,

amygdala, putamen, and caudate; a region with moderate SERT density, hippocampus, and a region with very low concentration of SERT, cerebellum, were included in the investigation. Anatomical boundaries were defined on the Hammersmith probabilistic atlas that has been shown to have high reliability for these regions (Hammers *et al*, 2003). Since the atlas is defined in Montreal Neurological Institute (MNI) space, the SPM MNI T1 template was spatially normalized with SPM5 to the coregistered individual MRI image and the deformation parameters applied to the probabilistic atlas to delineate the ROIs on the individual coregistered MRI. This normalized atlas was resliced to the dimensions of the PET images and fused with the individual gray matter map to obtain the time course of the average regional activity from gray matter only.

Finally, cerebellar gray matter was used as a reference region because of the comparatively low density of 5-HTT (Kish *et al*, 2005). To avoid the spillover from the occipital cortex, the reference region was carefully delineated in cerebellar gray matter and only up to five to eight slices were included leaving a margin of several millimeters around to the outer borders of the cerebellum (Hirvonen *et al*, 2007; Parsey *et al*, 2006). Final cerebellar region consisted on average of 11,071 voxels (± 1,134) each with dimensions 2.096 × 2.096 × 2.425 mm³.

Generation of Plasma Input Function

Arterial whole blood activity was monitored continuously for the first 28 minutes of the scan with a bismuth germanate coincidence detector. Ten discrete arterial blood samples were taken at 3, 9, 15, 21, 28, 35, 42, 50, 70, and 90 minutes into heparinized syringes. The activity concentration of the whole blood and the plasma was measured. Eight plasma samples per scan (at 3, 9, 15, 21, 35, 50, 70, and 90 minutes) were analyzed for metabolites using a semiautomated system with online solid-phase extraction followed by reverse-phase chromatography with online radioactivity and UV detectors and integration system. The amount of [¹⁴C]-DASB and its radioactive metabolites at a given time point were calculated from the decay-corrected integrated radio-chromatogram and from the levels of radioactivity in the solid-phase extraction eluate and expressed as a percentage of total plasma sample injected. Detailed information on data extraction and processing is reported in the original reference (Hinz *et al*, 2008).

Data Quantification

The proposed method of quantification for the calculation of BP_D is RS-ESA (Turkheimer *et al*, 2003), a Bayesian development of ESA that does not rely on the nonnegativity constraints of ESA and produces robust estimates of volumes of distribution for both plasma and reference modelling. RS-ESA reaches an effective compromise between the reliability of the estimates obtained by compartmental models with the flexibility of SA that does not require a predefined compartmental structure. When using a plasma input function, RS-ESA produces as output the total volume of distribution of the ROI V_T and BP_D

values can then be calculated as from equation (6). The numerical implementation of RS-ESA adopted standard parameters for ROI analysis, that is a 100 basis functions logarithmically spaced in the 10^{-4} s^{-1} to 1 s^{-1} range with the expected standard error of the target V_T set at 1%.

For comparison, we used standard compartmental modelling. For the case of a plasma input function, a two tissue/four rates compartmental model with an additional blood volume component (labelled '4k-bv') was used. The four rate constants K_1 , k_2 , k_3 , and k_4 describe, respectively, the forward and backward transport of the tracer from plasma to the free cellular space (that includes the nonspecific binding space) and association and dissociation to the specific target. Once the rate constants have been estimated, the volume of distribution can be calculated as:

$$V_T = \frac{K_1}{k_2} \times \left(1 + \frac{k_3}{k_4}\right) \quad (13)$$

For the compartmental case with a reference input, the SRTM was adopted. In SRTM, the tissue radioactive concentration $c(t)$ is expressed as a function of the reference region concentration $c_{\text{REF}}(t)$ as:

$$c(t) = R \times c_{\text{REF}}(t) + \left(k_2 - \frac{k_2 \times R}{1 + BP_D}\right) \times c_{\text{REF}}(t) \otimes \exp\left(-\frac{k_2 \times t}{1 + BP_D}\right) \quad (14)$$

In equation (14), R is the ratio between the K_1 rate in the target and the reference, and k_2 is the efflux rate.

RS-ESA and the above-defined compartmental models were estimated using their implementation in CLICKFIT,

an in-house software toolbox for Matlab (version 6.5, The Mathworks, Natick, MA, USA).

Data Analysis

The analysis of the regional data was performed according to the following steps.

For the citalopram study:

1. Total volumes of distributions were calculated with the plasma input function using RS-ESA and compartmental modelling for both baseline and citalopram infusion.
2. The volumes of distribution were then used in Lassen plots to estimate the 'true' occupancy and nondisplaceable volume.
3. Using the cerebellum as a reference region, the BP_D was then recalculated using RS-ESA and SRTM for both baseline and citalopram infusion.
4. Occupancy was calculated as the average percentage BP_D reduction in the citalopram infusion study from baseline.
5. The occupancy obtained at step 4 was corrected using corrections derived from equation (11) assuming a 30% displaceable volume in the reference as suggested by the PET literature (Parsey *et al*, 2006).
6. Differences in occupancies for four subjects obtained at step 5 were then compared with those obtained at step 2 and expressed as percent difference from the true value.

For the test-retest study:

7. BP_D was calculated with the plasma input function using RS-ESA to obtain the total volumes of distribution of the target regions and then applying equation (6) using the total volume of distribution of the cerebellum as V_{REF} .

Table 1 Citalopram study—plasma input modelling

	Amygdala	Brainstem	Caudate	Putamen	Hippocampus	Thalamus	Cerebellum
(a) Subject 1—Volumes of distribution (V_t) at baseline (□) and citalopram (■)							
RS-ESA	26.10	28.11	26.00	31.57	18.10	30.57	11.22
	17.44	17.67	16.06	19.01	14.30	17.11	11.14
4k-bv	30.40	30.30	30.40	36.10	21.90	35.80	11.80
	18.40	18.10	15.10	18.20	14.10	16.50	10.80
(b) Subject 2—Volumes of distribution (V_t) at baseline (□) and citalopram (■)							
RS-ESA	28.05	28.16	25.74	31.63	20.45	29.13	11.26
	17.07	16.90	15.39	19.67	14.27	17.48	9.14
4k-bv	32.00	31.60	30.10	37.10	23.60	35.30	13.50
	16.40	16.00	14.70	18.10	13.80	15.90	10.30
(c) Subject 3—Volumes of distribution (V_t) at baseline (□) and citalopram (■)							
RS-ESA	34.41	32.68	31.47	35.74	27.04	31.63	14.13
	17.17	16.76	16.79	19.31	16.05	17.78	10.62
4k-bv	37.30	35.00	39.50	43.10	31.10	38.50	17.10
	18.50	17.30	17.30	19.00	16.80	18.50	11.50
(d) Subject 4—Volumes of distribution (V_t) at baseline (□) and citalopram (■)							
RS-ESA	30.68	29.74	26.36	31.72	19.57	31.86	10.59
	15.12	14.50	11.53	17.04	13.11	16.64	7.30
4k-bv	29.50	25.40	22.50	28.50	17.40	28.40	9.30
	15.00	13.80	11.70	15.50	11.90	14.50	8.40

RS-ESA, rank-shaping regularization of exponential spectral analysis.

8. BP_D was calculated using a cerebellar input function using RS-ESA and SRTM to obtain the DVR values for the target region.
9. BP_D obtained at steps 7 and 8 was then used to obtain regional estimates of reliability assessed using the intraclass correlation coefficient (ICC) that was calculated using a one-way random model with subjects as random effects using SPSS (Release 18, IBM Corporation, Somers, NY, USA).

Results

Citalopram Study

Table 1 presents the total volumes of distribution for the four subjects estimated using the plasma input and calculated using either RS-ESA or the compartmental model 4k-bv. Volumes V_T were generally comparable across the two methodologies with the compartmental technique producing higher values for the baseline condition. This is in keeping with the volumes V_T produced by compartmental modelling being more skewed (log-normally distributed) the larger the values such as in the placebo condition, while RS-ESA produces normally distributed outcomes. The difference then disappears for the lower volume estimates in the citalopram experiment.

The regional volumes V_T were then used into Lassen plots to calculate the occupancies Occ and nondisplaceable volume V_{ND} . Figure 2 illustrates the plots for the four subjects that utilize volumes V_T estimated through RS-ESA. Because of the higher volumes produced by compartmental modelling in the baseline condition, the apparent occupancies estimated by RS-ESA were lower by ~9% in absolute terms than the one estimated by the 4k-bv model. By using the estimated V_{ND} , one can calculate the displaceable volume in the cerebellum of the four subjects that were, in percentage, 0%, 35%, 37%, and 44% (mean: 29 ± 19.7). These volumes were very comparable to those obtained from compartmental modelling (0%, 33%, 42%, and 42%, mean: 29.7 ± 20.4).

Table 2 shows the BP_D obtained by using the cerebellar TAC as input for both RS-ESA and SRTM for the two conditions, baseline and exogenous ligand, for the four subjects. The mean percentage change in BP_D after citalopram across ROIs was then used as an estimate of occupancy Occ . The occupancy values are shown in Table 3. From left to right, the values displayed are the occupancies estimated by BP_D , that are biased, the same occupancies that were bias-corrected using the tabled values demonstrated in Figure 1 using an *a-priori* value for the displaceable volume in the cerebellum of 30% and, finally, the occupancies obtained by plasma modelling that are here considered the standard. This is repeated for both RS-ESA and SRTM that are compared with the relative plasma methods.

Comparison between reference and plasma models shows that reference models, as expected, underestimated Occ on average by 14.2% (RS-ESA) and

Figure 2 The figure illustrates an example of the Lassen plots utilized to estimate occupancies in the four subject utilizing the equation $(V_1 - V_2) = (Occ \times V_1) - (Occ \times V_{ND})$ where V_1 and V_2 are, respectively, the regional volume of distributions at baseline and after citalopram infusion. In this case, the volumes of distribution were estimated using rank-shaping regularization of exponential spectral analysis (RS-ESA).

Table 2 Citalopram study—reference input modelling

	Amygdala	Brainstem	Caudate	Putamen	Hippocampus	Thalamus
(a) Subject 1— BP_D at baseline ($\square$) and citalopram ($\blacksquare$)						
RS-ESA	1.63	1.99	1.46	2.02	0.88	2.10
	0.69	0.59	0.54	0.78	0.44	0.64
SRTM	1.57	1.60	1.59	1.18	0.84	2.28
	0.59	0.59	0.38	0.70	0.43	0.49
(b) Subject 2— BP_D at baseline ($\square$) and citalopram ($\blacksquare$)						
RS-ESA	1.73	1.55	1.53	1.89	1.09	2.01
	0.96	0.75	1.20	0.92	0.44	0.83
SRTM	1.87	1.86	1.33	1.80	1.63	1.93
	0.57	0.56	0.49	0.51	0.38	0.60
(c) Subject 3— BP_D at baseline ($\square$) and citalopram ($\blacksquare$)						
RS-ESA	1.73	1.37	1.30	1.60	0.99	1.23
	0.56	0.54	0.53	0.78	0.45	0.67
SRTM	1.44	1.29	1.28	0.72	0.89	1.19
	0.52	0.51	0.47	0.52	0.44	0.57
(d) Subject 4— BP_D at baseline ($\square$) and citalopram ($\blacksquare$)						
RS-ESA	1.91	1.65	1.45	1.96	0.81	1.96
	0.86	0.77	0.62	1.25	0.59	1.13
SRTM	1.98	1.64	1.32	1.92	0.98	2.01
	0.81	0.68	0.47	0.57	0.49	0.84

RS-ESA, rank-shaping regularization of exponential spectral analysis; SRTM, simplified reference tissue model.

Table 3 Citalopram study—occupancies

	RS-ESA Ref.	RS-ESA Ref. corr.	RS-ESA plasma	SRTM	SRTM corr.	4k-bv plasma
Subject 1	0.620	0.690	0.650	0.615	0.682	0.715
Subject 2	0.478	0.559	0.524	0.699	0.766	0.702
Subject 3	0.564	0.641	0.640	0.530	0.610	0.713
Subject 4	0.452	0.535	0.614	0.601	0.678	0.673

RS-ESA, rank-shaping regularization of exponential spectral analysis; SRTM, simplified reference tissue model.

14.1% (SRTM). After bias correction, the underestimation on average is reduced to 0.0% (RS-ESA) and 2.7% (SRTM).

Note that, in the case of subject 1, bias correction causes an overestimation in Occ for RS-ESA but not for SRTM. This is due to the lack of cerebellar displaceable volume ($V_T = V_{ND}$) for this individual for whom the cerebellum is a 'proper' reference region.

Finally, to assess the sensitivity of the methodology, the corrected occupancies for RS-ESA were calculated for a range of values of the *a-priori* parameter p . On average for the four subjects, the use of $p = 10\%$ or 20% produced an underestimation, respectively, of 8.6% and 6.0% while using values of $p = 40\%$ or 50% caused an overestimation of 0.2% and 2.8%, respectively; average biases had an SD of ~ 0.09 .

Test-Retest Study

The aim of this section of the work was to investigate the reliability of the RS-ESA for the direct estimation of BP_D .

First, BP_D values were obtained by RS-ESA with plasma input function by subtracting from the target V_T the V_T of the cerebellum. These values were then compared with the BP_D obtained by RS-ESA using the cerebellar TAC as input. The aim was to establish the numerical consistencies of the reference methodology, that uses an input function sampled in a tissue with displaceable binding, with the results of the same methodology with plasma input.

Results are illustrated in Figure 3 that demonstrates a very tight linear correspondence between plasma and reference input derived values, whereas $R^2 = 0.989$ and the regression coefficient is equal to 0.965. In the same figure, the Bland-Altman plot does not show any trend of bias against mean value and the bias is negligible (0.042).

Finally, the reliability of the estimation of BP_D with RS-ESA with cerebellar input was quantified using the ICCs and compared with RS-ESA with plasma input and the SRTM. Results are shown in Table 4. Reproducibility of RS-ESA using a reference input was always higher than those obtained with SRTM, which did not cope well with the cerebellar TAC as input. The reliability of RS-ESA with reference input was also high (ICCs > 0.9) and comparable to those obtained with plasma input in all regions but amygdala and the brainstem. In these ROIs, the reliability of SRTM was also quite low.

Discussion

This work had two objectives. First, to demonstrate the potential use BP_D for the quantification of ligand

studies and studies with pharmacological challenges in particular. Second, to establish the numerical consistency and reliability of RS-ESA in obtaining BP_D estimates.

Figure 3 (A) Rank-shaping: regression of plasma over reference BP_{REF} . (B) Rank-shaping: Bland-Altman plot of plasma over reference BP_{REF} . The plot illustrates the relation between the BP_D estimates obtained by rank-shaping regularization of exponential spectral analysis (RS-ESA) using either a reference or a plasma input. Note that both values BP_D are underestimates of BP_{ND} since the cerebellar reference used has specific binding. The regression analysis (A) demonstrates that the relationship is tightly linear ($R^2 = 0.989$, regression coefficient is equal to 0.965). The Bland-Altman plot (B) demonstrates negligible bias and no relationship between bias and range.

In the citalopram study, the use of a reference TAC with bias correction reduced the underestimation from 14.2% to 0% (for RS-ESA) and from 14.1% to 2.1% (for SRTM) by using an *a-priori* estimate of specific volume of distribution of $\sim 30\%$. Note that use of the individual-specific values of distribution obtained from the individual subjects using the plasma data would have brought the individual biases to $\sim 0\%$. Although it is not realistic to foresee a scenario where the same individuals undergo a number of PET studies using different selective serotonin reuptake inhibitors it is, however, worth mentioning that this methodology allows the acquisition of data from several doses in one subject which usually is not possible with methods based on the arterial input function as repeated arterial cannulations are typically restricted to a maximum of two. Although the number of subjects considered in the study was limited to four, the results were quite consistent, particularly when RS-ESA was used. The fact that one subject did not demonstrate any displacement effect in cerebellum may also be consistent with the current knowledge on the serotonergic system; serotonin receptors and transporters are transiently expressed in the human cerebellum during early childhood and usually level off until adolescence but do persist in some individuals (Hirvonen *et al*, 2007).

The demonstration that PET drug occupancy studies can be analyzed using reference region kinetic approaches when a region devoid of specific binding is not available is of practical relevance as it allows the completion of drug occupancy studies on normal volunteers and patients without arterial cannulation and subsequent blood/plasma and metabolite measurements. This is a particular advantage for studies of neuropsychiatric disorders where patients may be unable to tolerate arterial cannulation.

The second part of the work, the test-retest study, demonstrated that RS-ESA is a sound and reliable numerical methodology for the calculation of BP_D . The tight linear relationship between BP_D calculated either with plasma or with a reference input and the lack of bias substantiated the ability of RS-ESA to cope with a multicompartmental reference. Furthermore, the very high ICCs, very similar to those of the plasma kinetic methodology, further motivate the use of BP_D as a quantification tool of general applicability in ligand PET studies. The ICCs were low in the brainstem for both RS-ESA and SRTM; excluding motion as a possible explanation (the plasma input

Table 4 Test-retest study—intraclass correlation coefficients

	Amygdala	Brainstem	Caudate	Putamen	Hippocampus	Thalamus
RS-ESA plasma	0.956	0.977	0.877	0.957	0.978	0.978
RS-ESA ref.	0.521	0.322	0.971	0.986	0.993	0.936
SRTM	0.567	−0.285	0.764	0.269	0.418	0.795

RS-ESA, rank-shaping regularization of exponential spectral analysis; SRTM, simplified reference tissue model.

ICCs were very high), the most likely reason for the observed ICCs is the tissue heterogeneity of this ROI. The brainstem, as defined in the Atlas used in this study, included a number of nuclei with possible diverse perfusion rates as well as the raphe where the concentration of the SERT is substantial. Given that the cerebellar TAC is far away from an ideal pulse, and given that the aim of any deconvolution technique is to estimate the impulse response function of the tissue, any numerical minimization is likely to struggle to fit the target TAC when it contains a number of kinetic diverse components. Indeed, amygdala and brainstem have been previously reported to be problematic in methodological work on reference modelling (Anderson *et al*, 2007).

We conclude with a note of caution. The validation of an appropriate methodology for the calculation of BP_D does not imply the systematic use of a reference with displaceable fractions in any radioligand PET study. It is important to emphasize that the selection of an anatomical region as reference tissue, whatever the application, should be supported by ancillary evidence that the tissue in that anatomical region is not affected by the pathological processes under investigation.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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