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Reliability of bulky DNA adducts measurement by the nuclease P1 ^{32}P-post-labelling technique

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Abstract

The aim was to assess the reliability of bulky DNA adducts measurement by means of the ^{32}P-post-labelling assay. The research design consisted of an intramethod reliability study. Buffy coats from 41 subjects were used to obtain two aliquots of 1–5 μg DNA for each subject; bulky DNA adducts were measured using the nuclease P1 ^{32}P-post-labelling technique. The reliability of the measurement was assessed by means of the intraclass correlation coefficient (ICC), the distribution of the differences between the two measurements and the limits of agreement. The estimated ICC was 0.977, with a 95% confidence interval between 0.921 and 0.977. The limits of agreement were ± 0.44 (DNA adducts per 10^8 nucleotides). Only three subjects had differences lying out of such limits. Bulky DNA adduct levels measured by the ^{32}P-post-labelling technique showed good reliability. Only one measurement is needed to use DNA adducts as a biomarker of exposure and, possibly, cancer risk. Besides, as a validation analysis, ^{32}P-post-labelling measurements can be repeated in only 20–30% of samples.

Keywords: *Reliability, DNA adducts, post-labelling technique*

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are formed during the incomplete combustion of organic matter and are released in the form of complex mixtures. Sources of PAHs include several industrial activities, vehicle exhausts and tobacco smoking (Besarati Nia et al. 2002). PAHs may also occur in foods by cooking them at high temperature, or as result of environmental pollution (IARC 1984, Bostrom et al. 2000, Jakszyn et al. 2004). Among non-smokers without a defined environmental source of exposure, diet may be the main contributor to total PAH body burden

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(Goldman and Shields 2003). Several PAHs have shown genotoxic or mutagenic effects in cell systems and are tumorigenic in animal models, but their data concerning carcinogenicity in humans are scarce (IARC 1984).

PAHs constitute one of the major classes of carcinogens (IARC 1984) that can bind covalently to DNA, referred to as DNA adducts. If unrepaired they may, eventually, result in the production of mutations and tumour initiation (Bostrom et al. 2000, Poirier et al. 2000). Most PAHs are activated to the diol-epoxide metabolite, which form bulky adducts preferentially at the exocyclic amino group of guanine. DNA binding appears to be linear over a wide dose range in *in vitro* systems. For example, a linear dose–response relationship between increased doses of benzoa(a)pyrene [B(a)P] and the formation of DNA adducts has been reported for human fibroblasts (Van Schooten et al. 1997). Instead, the relationship between B(a)P and DNA adducts has been found to be linear at low doses but sublinear at high doses in humans occupationally exposed to environmental carcinogens, showing that the *in vivo* formation of DNA adducts tends to reach a kind of saturation point/plateau at higher exposure levels (Peluso et al. 2001).

Past years have known the development of a number of techniques for the analysis of DNA adducts. The most used laboratory techniques are immunoassays, immunohistochemistry, ^{32}P-post-labelling, fluorescence and phosphorescence spectroscopy, electrochemical detection and mass spectrometry (MS). The ^{32}P-post-labelling assay has been one of the most widely applied methods for the detection of DNA adducts because of its high sensitivity and the requirement of small amounts of DNA. Even though it does not allow for structural identification, unless combined with preparative techniques or appropriate known DNA adduct standards (Peluso et al. 1991, Shields et al. 1993a,b, Munnia et al. 2004), it is especially suitable for detecting bulky, hydrophobic adducts, such as those induced by PAHs and arylamines (Peluso et al. 1991, Hemminki et al. 2000, Poirier et al. 2000, Peluso et al. 2001).

Although the ^{32}P-post-labelling assay has been widely used and standard protocols have been proposed (Phillips et al. 1999, Peluso et al. 2001, Phillips 2002), it is a complex technique, requiring a careful set up of procedures. Because of that, the quality of the measurement should be assessed at each specific application. The purpose of this work is to show the results of a reliability study of DNA adducts measurement by means of the nuclease P1 ^{32}P-post-labelling technique, an assay mainly effective at detecting PAH DNA adducts (Gupta and Earley 1988, Peluso et al. 1997, Peluso et al. 2001), within an epidemiological study, by comparing two series of measurements carried out in two aliquots of DNA extracted from one buffy coat sample of a group of subjects.

Material and methods

Blood collection and storage

The main study included a sample of 150 men and women aged 35–64 years from the Spanish cohort of the EPIC study (Bingham and Riboli 2004). Apart from information of the usual diet and lifestyle factors, a blood sample was collected from each subject at the time of enrolment. A 0.5 ml straw of buffy coat stored in liquid nitrogen was used.

Chemicals

Ribonuclease A, ribonuclease T1, proteinase K, micrococcal nuclease, spleen phosphodiesterase and nuclease P1 were purchased from Sigma Chemical (St Louis, MO, USA) and Sigma-Aldrich Chemie (Steinheim, Germany). Carrier free [γ-^{32}P]ATP (3000 Ci mmol^{-1}) was from Amersham (Amersham, UK). T4-polynucleotide kinase (PNK) was from Epicentre Technologies (Madison, WI, USA). Poly(ethyleneimine) (PEI) cellulose thin layer chromatography (TLC) sheets were from Macherey-Nagel (Postfach, Germany) and Merck (Darmstadt, Germany). All other chemicals and biochemicals were of analytical grade and used without further purification.

DNA extraction and purification

DNA was extracted from 100–150 μl buffy coats in the Florence laboratory. DNA was isolated and purified using a method that requires digestion with ribonuclease A, ribonuclease T1 and proteinase K, extraction with saturated phenol, phenol/chloroform/isoamyl alcohol (25:24:1), chloroform/isoamyl alcohol (24:1) and ethanol precipitation (Reddy and Randerath 1990). DNA was gently dissolved in distilled water and its concentration and purity were determined using a Beckman DU 800 spectrophotometer. Coded DNA was stored at −80°C until laboratory analysis.

^{32}P-DNA post-labelling technique

Leukocytes DNA adducts were blindly analysed using the nuclease P1 modification of the ^{32}P-DNA post-labelling technique in the Florence laboratory (for details, see Peluso et al. 1991, Gupta 1993, Phillips et al. 1999, Palli et al. 2000). Coded DNA samples (1–5 μg) were initially digested to normal and adducted 3′-mononucleotides with micrococcal nuclease (0.46 U) and spleen phosphodiesterase (0.0174 U). After treatment of DNA samples with 5 μg nuclease P1 for 30 min at 37°C, the hydrolysate enriched in adducted nucleotides were then labelled by incubation with 25–50 μCi carrier-free [γ-^{32}P]ATP (3000 Ci mM^{-1}) and 10 U T4 polynucleotide kinase. Detection of bulky DNA adducts was carried out by multidirectional PEI-cellulose TLC. Plates were first developed overnight using 1 M sodium phosphate (pH 6.8) then the resolution of the bulky DNA adducts was achieved with a two-dimensional chromatography (15) using 4 M lithium formate, 7.5 M urea (pH 3.5) and 0.65 M lithium chloride, 0.45 M Tris base, 7.7 M urea (pH 8.0). The plates were finally developed overnight using 1.7 M sodium phosphate (pH 5.0).

Detection and quantification of ^{32}P-labelled DNA adducts and total nucleotides were performed by storage phosphor imaging techniques employing imaging screens from Molecular Dynamics (Sunnyvale, CA, USA). After exposure, the screens were immediately scanned using a Typhoon 9210 (Amersham). Software used to process the data was ImageQuant (v.5.0) from Molecular Dynamics. The levels of DNA adducts were expressed as relative adduct labelling (RAL) = screen response (volume) in adducted nucleotides/screen response (volume) in total nucleotides (Reichert et al. 1992).

All analyses were carried out blindly, and a standard, e.g. B(a)P DNA adducts, from liver of mice treated intraperitoneally with 0.5 mg B(a)P, was routinely included in the analysis. The average RAL levels of B(a)P DNA adducts were 27 ± 1.8 SE per 10^8.

The detection limit was 0.1 adduct per 10^9 normal nucleotides, as reported (Reddy and Randerath 1986, Beach and Gupta 1992). An aliquot of 1–5 μg DNA was used to measure bulky adducts; in 41 of the 150 subjects a second aliquot of 1–5 μg DNA was used to carry out a second independent measurement. Half of the duplicate samples were analysed on the same assay and the other half in separate experiments performed in different days.

Statistical analysis

The first step to compare the two measurements was the inspection of the bivariate plot and the adjusted regression line. As the overall measure of agreement between two measurements, we calculated the intraclass correlation coefficient (ICC); this coefficient takes values between 0 and 1, where 1 is perfect agreement (Armstrong et al. 1994). It is defined as a function of the components of variance and calculated by means of ANOVA techniques. There are several versions of the ICC, and the selection of the appropriate version depends on the study design. In our case, we dealt with a simple replication study (intramethod reliability), where there is no characteristic that distinguishes the first and the second measurement across subjects, and thus we analysed the data by means of a one-way random effects model. The exact 95% confidence interval (CI) was calculated from the ANOVA table based on the F distribution.

Repeatability was assessed by analysing the differences between the two measurements (Bland and Altman 1999). The two means were compared by a paired t-test; we then calculated the repeatability coefficient as $1.96(2s_w^2)^{1/2}$, where s_w^2 is the residual mean square from the ANOVA table. The limits of agreement can be calculated as plus or minus the coefficient of repeatability ($\pm 1.96(2s_w^2)^{1/2}$); it is assumed that the true mean difference is 0 and the 95% of the differences lie within the repeatability coefficient. We assessed graphically the dispersion of the differences as well as the relationship with the magnitude of the measurement by plotting the difference between measurements for each subject against their mean, with the limits of agreement as horizontal lines at the y-axis. The existence of a linear dependency between the differences and the magnitude of the measurement may be formally tested by comparing to 0 the slope of the regression line in this graphic.

Results

The mean of the first measurement (RAL1) was 0.995 (per 10^8) adducts, with a standard deviation 0.75, while the mean and standard deviation from the second measurement (RAL2) were 1.004 and 0.78, respectively. The paired t-test for comparison of two means had a $p = 0.81$. The joint distribution of the two measurements is shown in Figure 1.

The graph also shows the line of equality, where all points would lie if the two measurements had exactly the same value. The points representing the pair of values for each subject are reasonably well distributed around this line. The constant and slope of the regression line corresponding to these points are not significantly different from 0 and 1 respectively ($p = 0.17$). The good overall agreement between two measurements is also reflected by the high ICC of 0.957 (95% CI 0.921–0.977).

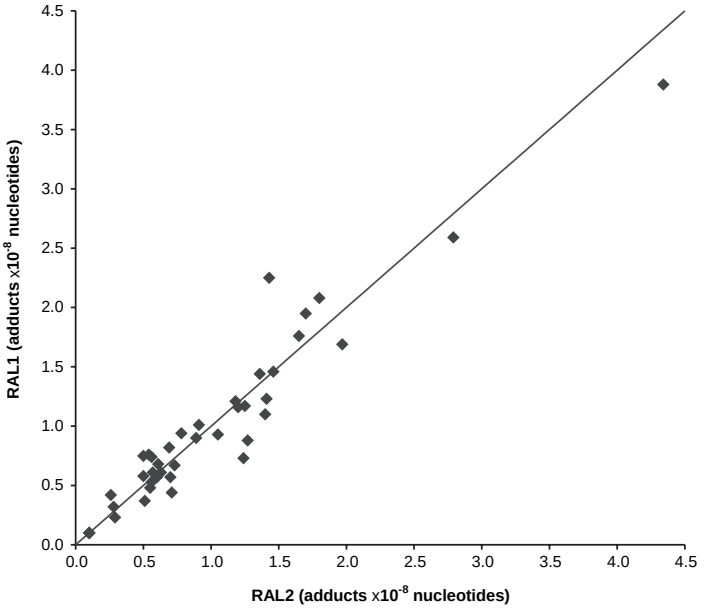

Figure 1. Plot of the two DNA adducts measurements (RAL1, RAL2) in 41 subjects, with the line of equality, corresponding to perfect agreement of the two measurements (RAL1 = RAL2). Regression equation: RAL1 = 0.076 + 0.915 RAL2. Test $a = 0$, $b = 1$: $F(2,39) = 1.83$, $p = 0.17$. RAL, relative adduct labelling, as adducts $\times 10^{-8}$ nucleotides.

The relationship between the differences and the magnitude of the measurement is shown in the Figure 2. The mean of the differences (RAL1 – RAL2) is -0.0088, non-significantly different from 0 as mentioned above ($p = 0.81$). The limits of agreement estimated from the repeatability coefficient are ± 0.44. Overall, the differences are symmetrically distributed around the theoretical value 0, with only three values lying out of the limits of agreement. There seems to be no dependence on the magnitude of the measurement estimated by the average of the two measurements, the slope of the adjusted regression line being non-significantly different from 0 ($p = 0.35$).

Discussion

Several circumstances may produce errors in the measurement of bulky DNA adducts by ^{32}P-post-labelling techniques (Beach and Gupta 1992, Phillips et al. 1999, Reddy 2000). Purification is an important step of the procedure; indeed, the method of DNA extraction has been reported to influence the levels of DNA adducts and a false-positive response could be due to high RNA contamination (Godschalk et al. 1998). On the other hand, post-labelling assay may underestimate the total levels of DNA adducts because of the inefficiency of some adduct labelling or loss of some adducts during enrichment procedures (Beach and Gupta 1992, Shields et al. 1993a,b, Phillips et al. 1999). In spite of these drawbacks, the technique has good sensitivity, with a detection limit of 0.1 adducts per 10^9 nucleotides (Reddy and Randerath 1986, Beach and Gupta 1992). Besides, a previous Interlaboratory Trial aimed at the standardization and validation of different ^{32}P-post-labelling assays has shown that the analysis of DNA adducts induced in *in vitro* from PAHs, such as B(a)P, may be quite

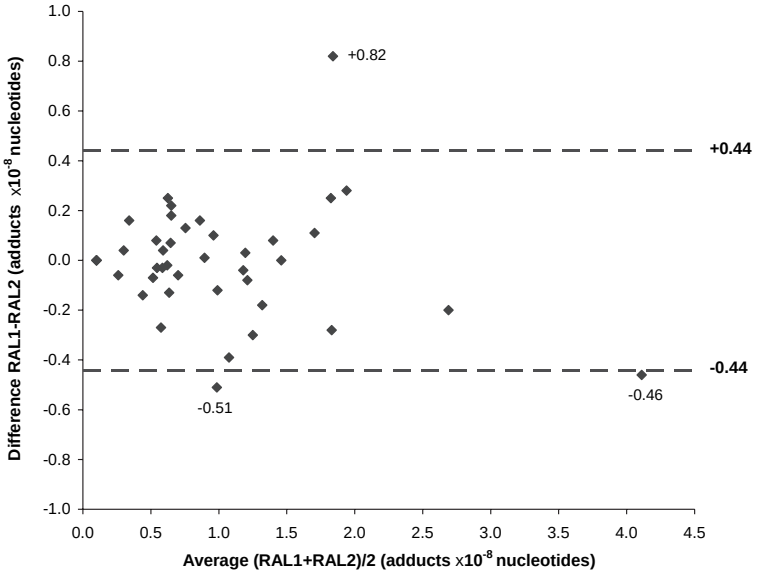

Figure 2. Plot of the difference between the two measurements (RAL1−RAL2) versus the average of the two values ((RAL1+RAL2)/2) in 41 subjects. The two horizontal lines correspond to the limits of agreement, calculated from the repeatability coefficient (±0.44) assuming that the theoretical mean of differences is 0. Regression equation: difference(RAL1−RAL2) = 0.036−0.455 average((RAL1+RAL2)/2). Test $b = 0$: $t(39) = -0.95$, $p = 0.35$. RAL, relative adduct labelling, as adducts $\times 10^{-8}$ nucleotides.

efficient, up to the 93% of the value obtained from measurement of the ^{3}H label in the DNA samples (Phillips et al. 1999).

According to our results, the nuclease P1 modification of the ^{32}P-post-labelling technique has also a good reliability. The term 'reliability' is generally used to refer to the reproducibility of a measure, i.e. how consistently a measurement can be repeated on the same subjects (Armstrong et al. 1994). Specifically, intramethod reliability is a measure of the reproducibility of an instrument, for instance, applied in the same manner to the same subjects at two or more occasions, or at two or more aliquots of the same blood sample. We estimated the reliability by the ICC = 0.957, consistent with high correlations from 0.71 to 0.98 observed in studies similar to ours (Palli et al. 2000, Peluso et al. 2000).

Most statistical tests used in our analysis assume normality of the variables, while the adduct measurements are rather right-skewed and normality can hardly be assumed. However, the methods applied are quite robust, and non-normal distribution of differences may not be as serious here as in other statistical contexts; moreover, non-normal distributions are still likely to have about 95% of observations within 2 standard deviations of the mean. Anyway, all the tests and estimates were also carried out by means of bootstrap techniques (Efron and Tibsirani 1993), and the main conclusions remain the same. For instance, the 95% CI estimated from 1000 repetitions were 0.88–098 for the ICC, and 0.43–0.45 for the limit of agreement, while the CI for the constant and the slope of the regression line of the two measurements always included 0 and 1, respectively.

The main interest of our results is related to the consequences for the main study: the good reliability observed means that we may rely upon a single measurement of

DNA adducts per subject to be used as potential biomarker. Besides, as validation analysis, ^{32}P-post-labelling measurements may be repeated in only the 20–30% of samples. This is important if we take into account that, without a gold standard, the only way to increase the reliability of an indicator is by taking repeated measurements. However, ^{32}P-post-labelling assay is a complex method, rather expensive and time-consuming; additionally, it requires DNA that could be used for other purposes. Thus, performing 191 measurements (including those belonging to the reliability study) instead of 300 or 450 if we would need two or three measurements for each subject may be seen as an important improvement of efficiency.

On the other hand, measurement errors, even if they are small, always affect precision of parameters, and they attenuate the estimates of association if they are non-differential. The correction of such error (de-attenuation) can be done by using the validity coefficient, which compares the observed measurement with the 'true'. However, when certain assumptions are met, the validity coefficient is equal to the square root of the reliability coefficient, estimated by the ICC (Armstrong et al. 1994). For instance, if the association of the measured adducts level with the occurrence of a given outcome is estimated by means of the odds-ratio (OR), the observed value may be corrected as OR($^{1/ICC}$). To apply such correction one must assume parallelism of the measurements, i.e. their errors are equal and uncorrelated. Strictly speaking parallelism cannot be tested without knowledge of the theoretical 'true' measure, but in practice it implies that the two measures have the same mean and variance, as it is our case; even when uncorrelated errors may occur, the reliability coefficient provide an upper-bound of the validity coefficient.

To conclude, the study shows that measurement of DNA adducts by means of the ^{32}P-post-labelling method has a good reliability, and only one measurement is needed to use DNA adducts as a biomarker of exposure and, possibly, cancer risk. Besides, as validation analysis, ^{32}P-post-labelling measurements may be repeated in only the 20–30% of samples. Furthermore, the estimated ICC may be used to de-attenuate associations based upon the adducts level measured in these subjects.

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