PREVALENCE OF BRONCHIAL REACTIVITY TO METHACHOLINE ASSESSED BY AN OPENING INTERRUPTER METHOD AND ITS CHARACTERISTICS IN A NORMAL GREEK POPULATION

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ABSTRACT

The bronchial responsiveness characteristics in a cohort of normal Greek population was investigated by the opening interrupter total resistance to breathing technique (Rt). We studied one hundred and seventy normal subjects, 100 male and 70 female (aged 38±8.5 and 35±7.5 respectively), non-smoking from normal families, ranging 18-60 years of age. These subjects had no respiratory symptoms, rhinitis or atopic history. A dosimetric cumulative inhalation of methacholine was used and the response was measured by the dose which increases baseline resistance by 100% ($PD_{100}Rt$) as well as by the dose response ratio (DRR). Rt was calculated from the pressure change at the end of the interruption period (end interruption Rt). Bronchial hyperresponsiveness (BHR) at a cut-off level of 0.8 mg methacholine exhibited 31 (18%, specificity 82%) normal subjects, 21 males and 10 females, while 3% of the total showed a response in the asthmatic range. No correlation were found between threshold dose or DRR and baseline airway calibre. Age and sex did not appear to influence the results. The method was well reproducible and showed good correlation with $PD_{20}FEV_1$ (r=0.76), with relatively narrow limits of agreement at -1.39ìmol and 1.27ìmol methacholine respectively. Rt-DRR was statistically far more sensitive than FEV_1-DRR in assessing the bronchoconstrictor responses, especially in subjects with abnormal reactions. Thirty eight percent of the subjects showed a plateau effect while their Rt-DRR was statistically 2.5 times less to the rest of the subjects with normal reactions. We conclude that the interrupter methodology is clinically useful and may represent an alternative to FEV_1 in situations when forced expirations cannot be performed. Since asymptomatic bronchial hyperresponsiveness is not a rare occurrence, further prospective studies are required in order to assess the epidemiological details of such a condition.

INTRODUCTION

It has been found that measures of bronchial airway responsiveness exhibit a continuous distribution in the general population, with asthmatic patients showing bronchial hyperresponsiveness (BHR) at the lower one tail of the curve and with some overlap (1). BHR defined as a provocative concentration of methacholine causing a 20% fall in forced expiratory volume in one second ($PC_{20}FEV_1$) has been found in as many as 23% of the general population at a cut-off value of 2 mg/ml (2). Other epidemiological studies have shown that as many as 11% of children who have never had any respiratory symptoms, have airway hyperresponiveness (3). All these studies included asthmatic patients or made no mention of smoking history or familial history of asthma; factors which have been shown to be associated with airway excitability (4,5). Community surveys have also shown a high prevalence of hyperresponsiveness in young asymptomatic individuals and there appears to be an inherited component (6,7). Malo et al, in a cohort of normal, non-atopic, non-smoking individuals from families without a history of asthma, found BHR defined as a $PC_{20}FEV_1$ of <16 mg/ml, in 8% of the subjects (8). In various other studies BHR in small numbers of normal subjects ranged from 3% to 10.4% (9,10). However, the methods used in the above studies are relatively insensitive (11). Attempts have been made to increase sensitivity by assessing BHR either by the provocative dose producing a 10% fall in FEV_1 or using flows at low lung volumes measured from a partial flow-volume curve, but mainly asthmatics have been studied thus far (12). Furthermore there are some special problems, such as a limited cooperation, fatigue and dizziness due to repetitive forced breathing manoeuvres, which limit the ability to assess lung function during challenges incorporating measurement of forced expiration. In addition a deep inspiration, as it occurs during an FEV_1 procedure, can either

cause transient bronchodilation or bronchoconstriction (13,14). Determining BHR using a technique which measures airways resistance is less influenced by inspiratory and expiratory efforts (15), and is more sensitive to small changes in bronchoconstriction, which make it more suitable for research studies in normal subjects in whom the response to bronchoconstrictors is limited (16). The total resistance to breathing (Rt) determined by an opening interrupter method has been found a useful means of assessing flow limitation during tidal breathing(17) as well as reproducible and suitable for diagnostic purposes in the detection and exclusion of asthma(18,19) but data concerning its operative characteristics in normals are lacking. Therefore we conducted a prospective cross-sectional survey using Rt methodolody in order to assess the features of bronchial reactivity in a large cohort of healthy non-atopic, non-smoking subjects from families without a history of asthma.

SUBJECTS AND METHODS

SUBJECTS

The study was conducted in Athens at the Department of Thoracic Medicine of a 500-bed hospital which is a tertiary referral center for respiratory disease and 200 subjects were initially enrolled, in accordance with the sample size estimation criteria (see later). All were healthy public employees and civilians who came either because of a routine yearly check-up or pending an evaluation for future engagement. This population corresponds to a middle class socio-economic status, all were of Greek origin living in the Greek countryside or at the outskirts of Athens, areas with no or low levels of air pollution. The routine check-up consisted of a history and a complete physical examination, complete blood count and chemistries, chest X-ray and spirometry.

All eligible volunteers were further examined for respiratory symptoms, determined from standardised questions of the ATS-DLD questionnaire(20). We included some additional questions such as: 1) do you suffer from perennial or seasonal rhinitis?, 2) did you or do you have infantile eczema, urticaria, migraine?, 3) does your father, mother, brother(s), sister(s), child(ren), have a history of asthma?, 4) were you ill from an upper respiratory tract infection in the previous 2 months?, 5) are you taking any kind of medication? A positive answer to any of those questions precluded further work-up. Nineteen subjects were excluded according to the above criteria, 10 more did not consent to participate and one subject reacted to the diluent control solution (0.6%), defined as a difference >30% baseline (19). The remaining 170 subjects, aged 18-60 years, consisted of 100 (59%) males and 70 (41%) females, having a distribution relative to sex and decade of age as shown in (Fig. 1). Predicted values for spirometry were those of ECCS (21). All participants were given information on the concept of the study and were asked to come the next morning avoiding tea, chocolate, cola and coffee drinks (22). A consent form was signed by all of the subjects studied.

METHODS

Routine spirometry was performed using a volume displacement water sealed spirometer (Gould 2400, Bilthoven, Holland) according to standardised guidelines(23). Volume-time and flow-volume curves were generated with the subject in a sitting position. Forced expiratory manoeuvres were performed in triplicate and the best effort was analysed. Subjects were instructed and vigorously coached to produce a forceful and prolonged expiration for at least 6 sec or as long as possible. Manoeuvres with too high extrapolation volumes (>5% FVC or 100 ml, whichever was greater) were rejected. Results were reported at ambient temperature, atmospheric pressure and saturated with

water vapour (ATPS). Methacholine bronchial provocation was performed after a rest of 10 min. The subjects were told the inhaled drug might result in bronchodilation, bronchocontriction or no response for the purpose of avoiding suggestion (24). We used three concentrations of methacholine (acetyl-beta-methylcholine bromide, No A2126, Sigma Chemicals, Saint-Louis, USA) 0.2%, 1% and 2.5% in a diluent of unbuffered saline, containing 1.5% benzyl-alcool as an antiseptic and conservative agent. Methacholine solutions were kept at 4°C for a maximum period of 20 days and were warmed before use to room temperature. Rt was measured by the opening interrupter method (25,26) (Bronchoscreen, Jaeger GmbH, Wurzburg, Germany). The unit has a combined provocation measuring head and the nebulisers produce an aerosol mist of a mass median aerodynamic diameter of 1.9ìm with 80% of droplets being less than 5.5ìm, delivered only during the first 0.6 sec of inhalation. The aerosol bolus is approximately 100 ml per breath and will transport 5.8ìl·breath^{-1} of solution at a set pressure of 1.6 bar (22.8 psi) (27). In this way and considering that during bronchial challenge the frequency is in average 20 tidal breaths·min^{-1}, its output is not much different from the well known Cockcroft nebulizer (1). Using the dosimeter method, variation in inhalation times from 1 sec to 5 sec does not influence methacholine deposition in the airways and the test outcome (28). Nebulizers were calibrated by the manufacturer and were rechecked by us (variation <5%) and were cleaned regularly (29). Rt was determined ten times and because repeatability increases with the patients habituation to the apparatus, the reported value is the average of the last five determinations. Rt was calculated using the formula:

$Rt = (P_{alv} / P_m) \times R_{ref}$, where the pressure in the alveolar compartment (P_{alv}) was obtained indirectly by measuring the mouth pressure after closing the shutter (end interruption pressure). This was chosen from previous work demonstrating that end interruption mouth pressure offers the best equilibration

with mouth pressure and sensitivity index during the challenge procedure (30). P_m was the pressure generated during free flow and R_{ref} was a fixed serial resistance. The shutter was triggered by an exhaled volume of 180 ml and opened after 100 msec closure. The triggering volume was determined by integrating the signal from a low resistance Lilly Pneumotach, which had a linearity of ±2% at a flow below 12 lit/sec. The pressure was determined by a differential transducer (Microswitch 87636, Honneywell). Before each challenge the interrupter was calibrated. A vent produced an airflow of 105 lits/min, which was led through the shutter and a calibrating resistance (0.10 kPa/litre/sec) and the determined Rt had to be within ±10% of the reference resistance. Under these conditions the repeatability of Rt is high (19). For replicated measurements on the same subject the within subject standard deviation was less than 0.05 kPa/lit/sec and the variation was independent of the magnitude of measurement. The method requires no cooperation from the subject and offers a direct instantaneous breath by breath monitoring of Rt even in the presence of mild to moderate bronchoconstriction (25,31), so it can be suitable for bronchial challenge procedures. Measurement was conducted during a relaxed steady state tidal breathing, while the subjects were asked to attain a target flow of at least 60 lit/min, guided by a column of green light emission diodes.

Basal Rt value was determined first over a period of 2 min in order to accustome the subject to the set up and get a steady state value and the same procedure was repeated with the diluent control solution. Then the subjects were challenged with a dose schedule consisting of 4-8-16 tidal breaths being taken at each of the three concentrations, until a final cumulative dose of 5 mg methacholine was delivered to the mouth or an increase in Rt of 100% ($PD_{100}Rt$) or greater was achieved. Doses were delivered at intervals of 1.5 min, while Rt was concurrently monitored during the procedure, thus avoiding

overprovocation. $PD_{100}Rt$ was chosen as the upper limit of normality for Rt values (1.64+SE) was in the range of +45.2%.

Results were assessed using two different estimates: 1) the threshold dose $PD_{100}Rt$ calculated by interpolation from the last two points of the cumulative semilogarithmic dose-response curve 2) the slope (dose-response ratio) of a line extending from the origin to the last point of the log-curve (Rt slope) (32). Interpolation between Rt saline value and $PD_{100}Rt$ was never performed.

The 10 days reproducibity of the $PD_{100}Rt$ method was investigated by randomly requiring every other subject to come again after one week for a second procedure. During this secod visit we also compared Rt with FEV_1 as a measure of response to provocation by estimating their respective $PD_{100}Rt$ and $PD_{20}FEV_1$ and slopes, FEV_1 determined after 30 sec of Rt measurement as previously described (19,33). At least two technically correct forced expiratory manoeuvres with a variation less than ±5% in the FEV_1 were obtained and the highest value was used for further calculations.

We measured again Rt after the FEV_1 procedure in order to ensure that lung function was stable before the next dose increment. A small transient bronchodilatation was noted by a fall in Rt by approximately 25%, which rapidly returned to previous stable levels in 0.5 secs (roughly after 10 tidal breaths).

Because a good correlation and agreement was found between $PD_{100}Rt$ and $PD_{20}FEV_1$, we used the established cut-off values of the latter, so that $PD_{100}Rt$ values below 0.8 mg methacholine were regarded as positive (34), 0.8mg to 2.0 mg as normal to borderline and above 2.0 mg (8.6 ìmol) as negative non-response reactions (35).

STATISTICAL METHODS

Regression analysis and correlation, the x^2 test, Shapiro-Wilk test for normality (36) and the non-parametric Mann-Whitney -U- /Wilcoxon Rank Sum test with normal approximation (37) were used for statistical analysis. The relative duplicate error was used to assess the test-retest reproducibility of the $PD_{100}Rt$ method (assuming a normal distribution), defined as the standard deviation of the differences divided by $\sqrt{2}$ after log transformation (approximates coefficient of variation) (38). Agreement between $PD_{100}Rt$ and $PD_{20}FEV_1$ was defined and calculated according to Bland and Altman (39). The difference between the first (x_1) and the second (x_2) method was plotted against their mean i.e. (x_1-x_2) against [(x_1+x_2) x 0.5] to determine whether the variation was related to the magnitude of the measurement. Given a mean prevalence rate of BHR from other studies of normal populations of 11% with a standard deviation of 7% and a type beta error of 0.20, a sample size of 198 normal subjects is required to detect a 9% prevalence of BHR (13). An alpha error cut-off value of 0.05 was used for statistical significance.

RESULTS

Table (1) shows the subjects' anthropometric data and baseline functional results, which were normal. Frequencies were higher for the 18-30 age group and with male preponderance (Fig 1). Mean values of vital capacity (VC), FEV_1 and maximal expiratory flow when 50% of the forced expiratory vital capacity (FVC) remains to be exhaled ($Vmax_{50}$) were higher in males by 16.3%, 14.7% and 2.5% in relation to females, respectively, while accordingly mean Rt values were found to be higher in females. Histograms of the frequency distributions of $PD_{100}Rt$ (threshold dose) in males, females as well as for all of the subjects studied are shown in Fig(2). We found that 21 males and 10 females (18%) of the total subjects studied, had bronchial hyperresponsiveness (specificity in a cohort of fit individuals 82%) with a mean value of

$PD_{100}Rt$ 0.57 mg (SD=0.24, SE=0.05, 95% confidence interval 0.47-0.76). These values were normally distributed (W=0.93, p=0.12), and there was no statistical significance between the two sexes (x^2=1.48, p=0.22, odds ratio=1.79, and unpaired t-test, t=1.61, p=0.12). Furthermore 5 of these subjects (3 men and 2 women ,3% of the total) were found to have BHR below 0.4 mg methacholine level (< 1.66 ìmol) which is frequently found in current symptomatic asthmatics (40).

No correlation was found between $PD_{100}Rt$ and baseline Rt (r=-0.14, 95% CI -0.03 to 0.31, p=0.10), $PD_{100}Rt$ and dose-response ratio (r= -0.06, 95%CI -0.11 to 0.24, p=0.48) and between dose-response ratio and baseline Rt (r=-0.07, 95%CI -0.10 to 0.21, p=0.37).

The remaining population showing normal bronchial responsiveness were further subdivided into two groups (one group considered as plateau with low maximal response), according to the threshold dose (sensitivity) and dose-response ratio (reactivity). Plateau was defined as a difference in Rt <40% after the delivery of three consecutive doses and/or a dose-response ratio <40% after a total cumulative dose of 4 mg, or a $PD_{100}Rt$ value >4 mg. These responses when extrapolated to the 100% level gave high incosistent values and so therefore represented censored data and were not kept for analysis, but values of the dose response ratio were calculated in the entire sample.

The slope of the subjects that showed negative results (>8.6 ìmol) was ten times lower to those with BHR, (mean±SE 67.52%±10.66 and 690%±705 respectively, p<0.001). Plateau response exhibited 66 (38%) of the subjects, 36 males and 30 females without statistical significance between them (x^2 of 0.81, p=0.36). Accordingly their slope was approximately 2.5 times lower and statistically significant to the rest of the subjects with negative but measurable reactions (mean±SE 30.1%±9.80 vs 75%±49.87, Mann-Whitney two tailed p by normal approximation p=0.024).

The subjects with BHR were then reexamined clinically and a complete personal and familial history was taken. Chest X-ray examination and immediate skin testing with 15 common aeroallergens (Allergofarma, Germany) were performed and total serum IgE was also measured but no atopic status was found nor a compatible asthmatic clinical picture. Furthermore venous blood was drawn and analysed by the recently introduced UniCAP FEIA system (Pharmacia,Sweden) for eosinophilic cationic protein (ECP) and a screening test for atopy (Phadiatop) (41). After a period of 3 months one of these subjects with $PD_{100}Rt$ of 60ìg and $PD_{20}FEV_1$ of 90 ìg, presented (also his son) with asthmatic symptomatology after an upper airway viral infection, so this person might have been an asthmatic who was a poor sensor of airways obstruction, confirming on the other hand older data that there is a 100% specificity for current asthma in subjects with a $PD_{20}FEV_1$ <100ìg (42). At that time he had an ECP of 27.8 ìg/l and was Phadiatop negative. The remaining subjects being Phadiatop and ECP negative (values <4 ìg/l) are under follow up and evaluation every 6 months.

There were no significant correlations between $PD_{100}Rt$ and age in years (r=0.10), baseline vital capacity (r=0.15), FEV_1 (r= 0.13), FEV_1% (r=0.17) or $Vmax_{50}$ (r=0.20) in percentages of the predicted. The mean value of each of these parameters were not significantly different between responders and non responders. The method was repeatable within ± 200 ìg for the $PD_{100}Rt$ performed in less than two weeks period and no adverse reactions due to methacholine were noted. The relative duplicate error was 8.33% and the intraclass correlation coeffiecient (between subject variance/total variance) was 93.4%. A good correlation was noted between $PD_{100}Rt$ and $PD_{20}FEV_1$ (Spearman's rank correlation r=0.76, 95% confidence intervals for r by Fisher's Z transformed= 0.53 to 0.88). $PD_{100}Rt$ values did not differ statistically from $PD_{20}FEV_1$, and a relative agreement between the two methods was noted (Fig.

3), but Rt slopes proved more sensitive in assessing the bronchoconstrictor responses than FEV_1 slopes (Table 2.).

DISCUSSION

Methacholine inhalation challenge has been well established as a procedure capable of demonstrating bronchial reactivity, since its clinical prediction has been found to be slightly better than chance alone (43). Studies of bronchial hyperreactivity in population studies restrict quantitative information to the 15-25% of subjects with a measurable $PD_{20}FEV_1$, whereas in the majority of cases the information is purely speculative: response or no response, posing particular problems in expression of the results (44). That is why we also used the dose-response ratio methodology, because it was found better than PD_{20} FEV_1 in epidemiological studies (45). This is the first systematic study were we applied more than one measure of responsiveness to study a large group of normal subjects by the shutter interrupter technique. If we estimate the size of the population at 150,000 where this sample was selected from, with an expected frequency of the factor under study at 50% and take 40%, as the level of the worst acceptable result, then our sample size of 170 subjects is at the 99% confidence level. This method although has been criticised as difficult to interpret in terms of mechanical properties of the respiratory system when compared to the forced oscillation technique (46), however recent studies evaluating its accuracy and sensitivity in measuring the response to bronchial challenge proved it to be a useful alternative (30). However, a small increase in alveolar pressure occurs during closure because of the elastic properties of the system. This additional increment in pressure is taken into account when calculating Rt. By this method equilibration of mouth pressure allows realistic measurement of respiratory system resistance, while with the forced oscillation method pressure is estimated from total compliance. The Rt was measured during expiration above FRC level, because resistance values hardly

change above this level and so therefore are not much influenced by variations in breathing efforts. Besides we did not corrected Rt values to lung volumes because the variability added by the determination of functional residual capacity (FRC) can abolish the benefit of equalization for lung volume and the correlation between respiratory resistance and FRC is not significant over the limited FRC range of healthy subjects (47). Furthermore we used for the first time in this study the $PD_{100}Rt$ threshold dose so that the possible small changes of functional residual capacity during challenge would not affect the repeatability of the Rt method and its correlation to FEV_1 (19,33). If a cut-off value of 400μg methacholine $PD_{100}Rt$ is set for the asthmatic range on the semilogarithmic dose-response diagram, then 3% of the studied normal population was found to be in this area. This is similar to the percentage found by Malo et al. (8) as well as to 2.5%, which represents the proportion of subjects beyond the 2 SD of the mean on one side of a normal distribution. Thus there is a small overlap between symptomatic asthmatics and normal subjects. Even though baseline airway resistance was normal, we cannot exclude that these normal "patients" might have milder degrees of airway wall thickening that could play a critical role in altering airway responsiveness. One of these persons, which are being followed appeared with symptoms of typical asthma after a relatively short time interval. Zhong et al. (48) using the $PC_{20}FEV_1$ method found that 45% of asymptomatic students with a positive test developed asthma in the following two years. If a limit of 800μg methacholine is taken to separate normal from abnormal reactivity, then a total of 18% of the subjects studied showed some degree of bronchial hyperreactivity. The significance of these findings cannot be overemphasised in view of the increased rate of decline in lung function found in subjects with bronchial hyperreactivity (49) . Plateau with a low maximal response defined as above, exhibited 38% of the persons, thus representing the least reactive portion of the sample. Similar results were reported by Seppala et al.,(50) where it was

found that 50% of the normals had no calculable $PC_{20}FEV_{1.}$. Some bias might have been introduced in our population selection, which was made up by subjects coming for a routine checkup, but we can argue our sample is representative of the normal population at large, since their spirometric indices were within the predicted reference values. No correlation was found in this study between BHR and baseline airway caliber, although Malo et al. found a slightly weak correlation when a more sensitive parameter , the provocative concentration causing 6% fall in FEV_1 (PC_6) was used. This difference might be due to the different method applied and to the selection of the study sample. Since this method occasionally can measure higher values due to averaging, glottis closing, tongue positioning and some other artefacts, extreme care was taken to exclude these values during the procedure. This was also achieved by simultaneous visual pressure curve tracing over time during each breath and reassuring the subject to breathe in a relaxed manner. Besides, at least half a minute stable resistance reading was required in order to be taken as represantative of Rt and frequent calibrations of the screen resistor were performed in order to assure that condensation effects did not interfere with the results. The limits of agreement between the described method and the classical $PD_{20}FEV_1$ method were found relatively small at -1.39ìmol and 1.27 ìmol respectively, so this method can be used as an alternative for challenge testing as it is simple and easy to perform and requires no patient cooperation. Some subjects exhibiting normal bronchial reactivity showed $PD_{20}FEV_1$ lower than $PD_{100}Rt$ values which although not statistically significant could be explained by the fact that during quiet breathing compression of the downstream segment does not play a major role in bronchial obstruction in contrast to FEV_1, so it seems that the shutter method is more “physiologic” and representative of airway wall caliber than FEV_1. In Summary, there is a wide distribution of bronchial responsiveness in our normal Greek population sample which has also been found in other European countries. Caution should be exercised

when interpreting the results of bronchial challenge tests in epidemiological surveys including normal populations because positive or borderline results are not uncommon. Further longitudinal studies are needed to better characterise these groups of subjects and assess their epidemiological profiles.

TABLE 1. Characteristics of the study population by sex

Variables	**Men**	**Women**
	n=70	n=100
age, mean (range) yr	35 (18-55)	38 (18-60)
height,mean(cm),(SE)	174 (0.78)	160 (0.71)
weight,mean(kg),(SE)	79 (0.9)	63 (0.9)
Resistance(Rt), mean(Kpa/l/sec),(SE)	0.24 (0.069)	0.29 (0.074)
VCin,mean (%predicted),(range)	111.5 (83-144)	95.2 (76-129)
FEV_1,mean (%predicted),(range)	107.9 (75-125)	93.2 (78-110)
FEV_1%,mean(range)	83 (77-92)	82 (75-90)
$Vmax_{50}$,mean (%predicted),(range)	83.5 (70-155)	81 (65-145)

VC in: inspiratory vital capacity
FEV_1: forced expiratory volume in 1 sec
FEV_1%: ratio of forced expiratory volume in 1 sec to forced vital capacity
$Vmax_{50}$: maximum flow at 50% of forced vital capacity

TABLE 2. Comparison of the methods described in text in terms of threshold dose (sensitivity) and dose-response ratio (reactivity), stratified according to BHR status.

Methods (x±SD)		A. subjects showing BHR		
Rt	$PD_{100}Rt$(mg)	0.57±0.20	dose response ratio(%)	690±705
FEV_1	$PD_{20}FEV_1$(mg)	0.72±0.66	dose-response ratio(%)	98± 90
p value			0.30	
		B. Subjects with normal measurable reactions (> 0.8 mg)		
Rt	$PD_{100}Rt$(mg)	3.42±3.10	dose response ratio(%)	74.86±49.87
FEV_1	$PD_{20}FEV_1$ (mg)	3.13±2.65	dose response ratio(%)	20±3.82
p value		0.6		0. 006

LEGENTS FOR FIGURES

Figure 1. Age distribution of the studied population, by sex.

Figure 2. Frequency distribution of $PD_{100}Rt$ (threshold dose) in males and females, according to cutoff values described in text. Values > 4 mg are derived from extrapolation and represent censored data.

Figure 3. Bland and Altman plot of the differences of the two methods against their mean value. Thirty subjects are not included due to a plateau effect by both methods. The limits of agreement $\bar{d}$-2s and $\bar{d}$+2s, are -0.334 mg (-1.39ìmol) and 0.306 mg (1.27ìmol) respectively. The 95% confidence intervals are -0.364 to -0.303 and 0.275 to 0.336 (mgs) respectively.Five normal subjects with theshold doses >4 mg (derived by near extrapolation) are also included in the graph.

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