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ASSAY OF UNDERIVATIZED NITRAZEPAM AND CLONAZEPAM IN PLASMA BY CAPILLARY GAS CHROMATOGRAPHY APPLIED TO PHARMACOKINETIC AND BIOAVAILABILITY STUDIES IN HUMANS

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SUMMARY

The assay procedure of underivatized, intact nitrazepam and clonazepam in human plasma is described, using gas chromatography with a support-coated open tubular column (OV-17), a solid injection system and electron-capture detection. Clonazepam is used as internal standard in the assay of nitrazepam and vice versa. Linear calibration curves after a single extraction step were obtained in the concentration range 10—100 ng/ml plasma, with standard deviations less than 4.9%. The sensitivity limit of the method is about 1 ng/ml plasma for both drugs.

The method was applied to pharmacokinetic and bioavailability studies of nitrazepam in humans. Seven healthy volunteers received two nitrazepam-containing tablet preparations (5 mg) and plasma concentrations were determined regularly from 15 min to 80 h following drug administration. The mean elimination half-life of nitrazepam was 27 h (range 13—34 h). Considerable intra-individual differences in peak level times between the two preparations were observed, whereas the extent of bioavailability was rather similar.

INTRODUCTION

Various methods for the determination of nitrazepam in plasma have been described in the literature, comprising thin-layer chromatography [1—5], photometric [6, 7], fluorimetric [8, 9] and radioactivity [10] measurements, high-pressure liquid chromatographic [11, 12] and gas chromatographic (GC) methods [13—20]. However, due to their lack of sensitivity or specificity, several of these methods cannot be applied to pharmacokinetic investigations when therapeutic doses are used. Of the GC methods some determine the benzodiazepines after acid hydrolysis as benzophenones [13, 17—19], which

leads to loss of specificity. Others require derivatization, e.g. methylation [15, 16] and trimethylsilylation [20]. In addition, laborious extraction procedures are often required for the isolation of the compounds from blood or plasma, which, in addition to chemical manipulation and long retention times, can make such methods very time-consuming.

It was our aim to develop a method for the assay of nitrazepam in plasma that could be applied to pharmacokinetic and bioavailability studies in man. The method should therefore be specific and sensitive and also rapid because of the great number of samples to be analyzed. The use of a so-called SCOT column (support-coated open tubular column) together with electron-capture detection proved to be suitable for our purpose. Next to nitrazepam, it was found that clonazepam could be analyzed equally well with this method.

MATERIALS AND METHODS

Cab-O-Sil (fumed silica, non-silanized, Grade M5; Carbot Corp., Boston, Mass., U.S.A. Duran 50 glass (Schott-Ruhrglas, Bayreuth, G.F.R.); benzyltriphenylphosphonium chloride (Aldrich, Milwaukee, Wisc., U.S.A.); Carbowax 20M (Chrompack, Middelburg, The Netherlands); OV-17 (Chrompack); distilled dichloromethane (Baker, Phillipsburgh, N.S., U.S.A.); distilled light petroleum (b.p. 40—60°, AnalaR grade; BDH, Poole, Great Britain); distilled ethyl acetate (Baker); nitrazepam (Hoffmann-La Roche, Basle, Switzerland); Mogadon (Hoffmann-La Roche) tablets containing 5 mg nitrazepam; Sameko tablets (Sameko, Katwijk, The Netherlands) containing 5 mg nitrazepam; clonazepam was a gift from Dr. T.B. Vree, Laboratory of Clinical Pharmacy, Radboud hospital, Nijmegen.

Extraction procedure

To 1.0 ml plasma in a centrifuge tube were added 25 μl ethanol containing 25.0 ng clonazepam (internal standard) and 1.0 ml borate buffer (0.2 *M*) pH 9.0. After homogenization the mixture was extracted twice with 5 ml light petroleum (b.p. 40—60°)—dichloromethane (1:1) on a Cenco whirlmixer for 20 sec. After centrifuging for 5 min at 2500 *g*, the upper organic layer was removed with a pasteur pipette and transferred to a conical evaporation tube. The solvent was evaporated to dryness at 50—60° in a flow of dry nitrogen on a water bath. The residue was dissolved in 50 μl ethyl acetate and 1—2 μl of this solution were brought onto the needle of the solid GC injection system. After evaporation of the ethyl acetate the residue was injected into the gas chromatograph. The whole extraction procedure is represented in Fig. 1.

Apparatus

A Hewlett-Packard Model 5713A gas chromatograph, equipped with a ^{63}Ni-pulse-modified electron-capture detector, was used. The solid injection system was a modified pyrolysis system (Becker, model 767) which has been used by Driessen and Emonds [21] for the determination of antiepileptic drugs. Temperatures: injection port, 250°; column, 230°, detector, 300°. Gas flow-rates: through the column 10 ml/min, argon—methane (95:5); auxiliary gas,

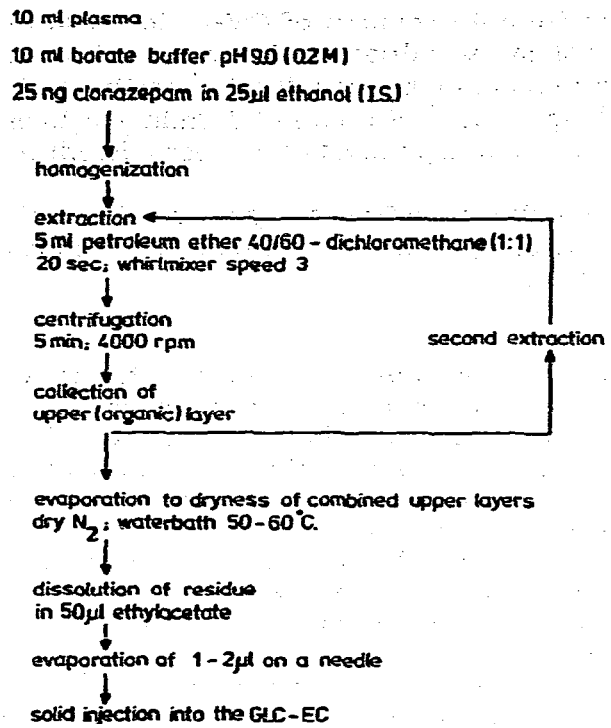

Fig. 1. Extraction scheme for the isolation of nitrazepam from plasma and the subsequent gas chromatographic determination.

argon—methane (95:5) was added at the end of the column to obtain a total flow-rate through the detector of 35 ml/min. Capillary columns were prepared using a Hupe and Busch glass-drawing machine. The glass column (Duran 50) had a length of 10 m, and I.D. of 0.40 mm and an O.D. of 0.80 mm. The column was cleaned with acetone and carbon tetrachloride. The support material (0.5 g Cab-O-Sil Grade M5) was deactivated with 2 ml of a 1% solution of benzyl triphenylphosphonium chloride (BTPPC) in dichloromethane. The excess BTPPC was removed by washing the support material two times with 10 ml of dichloromethane. After centrifugation the dichloromethane was decanted and the support material was suspended in 15 ml carbon tetrachloride by placing it in an ultrasonic bath for 15 min. The column was coated with this suspension at a rate of about 10 cm/sec. Before coating a small plug (± 40 cm) of carbon tetrachloride was brought into the column and immediately followed by the suspension in order to prevent blocking of the column. At the end of the column a dummy column (25% of the column length) was attached in order to prevent a sudden rise in the coating rate when the suspension starts to leave the column. After the suspension had left the column the flow through the column was increased for 3 h for drying and also to prevent droplets forming.

The column was then deactivated by coating dynamically with a 1% solution of Carbowax 20M in dichloromethane at a rate of 5 cm/sec. After drying,

the column was coated dynamically with a 3% solution of OV-17 in dichloromethane at a rate of 5 cm/sec. The column was dried for 3 h with a nitrogen flow of 20 ml/min. The column was conditioned overnight by starting with temperature programming from 50° up to 270° at a rate of 1°/min and then being kept at 270° overnight. A similar column preparation has been described previously [22].

For the identification of the compounds eluting from the gas chromatograph an LKB-2091 combined gas chromatograph—mass spectrometer equipped with a PDP-11 computer system was used.

Preparation of calibration curves

The concentration of nitrazepam in plasma was calculated with the aid of calibration curves prepared by adding known amounts of nitrazepam to 1.0 ml blank plasma. These standard samples were analyzed by the same procedure as described above and the ratios of the peak areas of nitrazepam to internal standard were plotted against the known concentrations of nitrazepam. The same procedure was followed for estimating the extraction yield of nitrazepam from plasma at various concentrations, except that clonazepam was used as an external standard (25.0 ng). The ratios found were compared to the ratios of standard amounts of the drugs. Calculation was carried out manually by calculating the peak area (peak height × peak width at half peak height). Before analysis of a sample series, calibration was always carried out using two plasma samples containing known concentrations of nitrazepam. Stock solutions of nitrazepam and clonazepam were stored in the refrigerator at 4° to avoid possible decomposition [23].

Human studies

Seven healthy male volunteers (aged 20—23 years, body-weight 64—86 kg) participated in the study after they had been medically examined. At an interval of two weeks each volunteer received one tablet containing 5 mg nitrazepam. Two different brands (Mogadon and Sameko) were used in a cross-over design. Subjects were instructed not to take other drugs or alcohol from 24 h before, until 24 h after, the beginning of the experiments. In addition they were not allowed to drive a motor vehicle for 48 h following drug administration.

After an overnight fast, at 9 a.m. the volunteers swallowed the intact tablet with 150 ml tap water. Initially they were asked to remain in an upright position for 15 min and then to lie down for at least 3 h. This procedure was undertaken in order to simulate as closely as possible the situation of taking a hypnotic drug and going to bed. No food, fluid or tobacco was allowed for 3 h after drug administration. Blood samples were taken at ¼, ½, ¾, 1, 1¼, 1¾, 2, 2½, 3, 4, 6, 8, 24, 32, 48, 56, 72, and 80 h from a forearm vein, for the first three hours of the experiment by means of a flexible venous cannula with injection valve. After three hours blood samples were taken by venous puncture. Blood clotting was prevented by adding a small drop of heparin solution (5000 I.U./ml) to the samples. After centrifugation the plasma samples were stored in the refrigerator at —20° until analysis.

RESULTS AND DISCUSSION

Assay procedure

Fig. 2 shows gas chromatograms of plasma extracts of plasma samples taken 1½ and 80 h after oral ingestion of a tablet containg 5 mg nitrazepam, as well as the gas chromatogram of a blank extract. There is no interference from endogenous plasma substances or metabolites and retention times are short. Clonazepam was chosen as an internal standard in the assay of nitrazepam and the two peaks are well separated at low and high concentrations.

Identification of the compounds eluting from the gas chromatograph was carried out by means of combined gas chromatography—mass spectrometry (LKB-2091 with PDP-11 computer system). Figs. 3 and 4 show the computer plots of the mass spectra of nitrazepam and clonazepam respectively. By comparison of these mass spectra with direct inlet mass spectra of the pure reference substances it can be concluded that nitrazepam and clonazepam both leave the gas chromatographic column unchanged, so that they are being determined in intact form.

According to the standard curve (Fig. 5) there is a linearity between the detector response (peak area nitrazepam/peak area clonazepam) and the concentration of nitrazepam between 10 and 100 ng/ml plasma. Also in Fig. 5 are given recovery values for the same concentration range. In spite of the short extraction time (20 sec) the recovery of nitrazepam from plasma is high: average of 92% (S.D. at each concentration 3.5% or less; $n = 4$). The reliability of the whole procedure is also reflected in Fig. 5, which shows the mean graph of three calibration graphs made on different occasions; the highest value for the S.D. was 4.9%. The present procedure can also be used

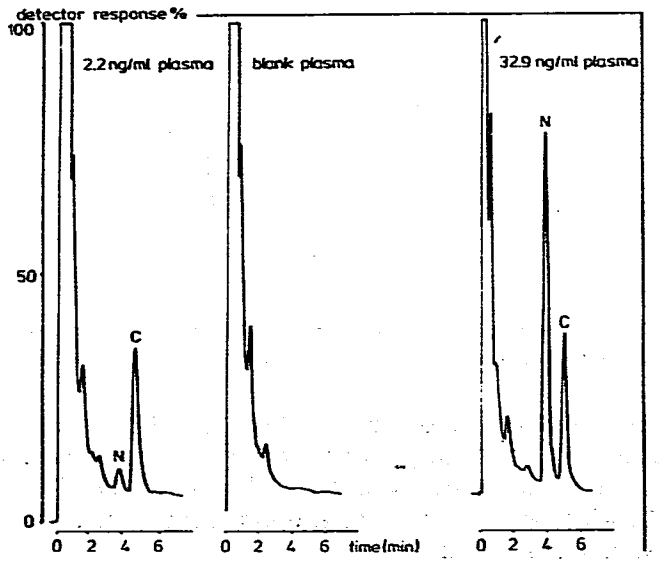

Fig. 2. Gas chromatograms of a 1-ml plasma extract obtained from a volunteer immediately before (middle), and 1.5 h(right) and 80 h (left) after, receiving 5 mg nitrazepam orally. N = Nitrazepam, C = clonazepam (internal standard, 25.0 ng/ml plasma).

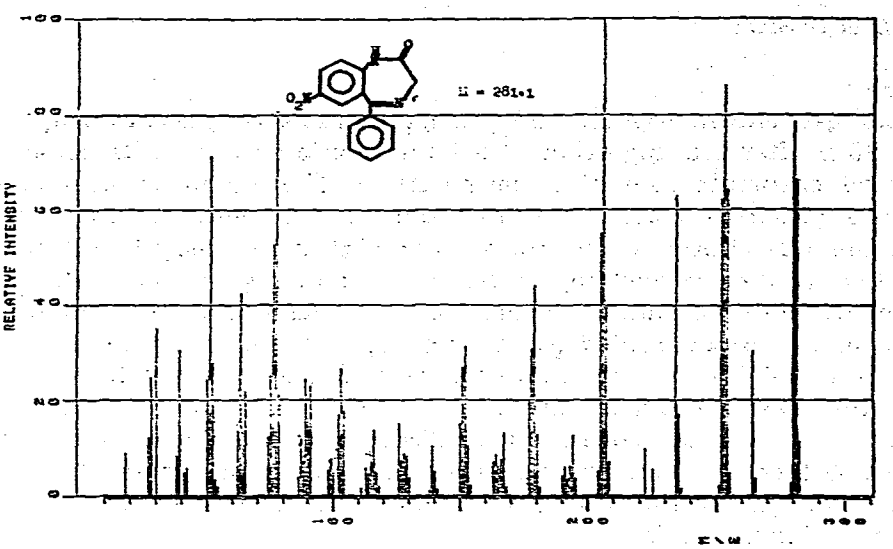

Fig. 3. Normalized electron-impact mass spectrum of nitrazepam obtained by applying a plasma extract to the LKB-2091 gas chromatograph—mass spectrometer.

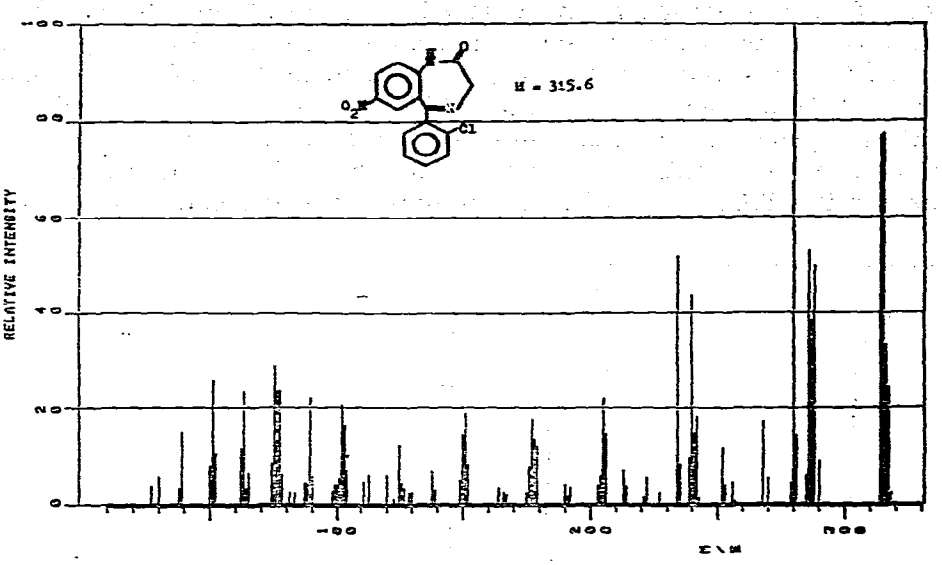

Fig. 4. Normalized electron-impact mass spectrum of clonazepam obtained by applying a plasma extract to the LKB-2091 gas chromatograph—mass spectrometer.

for the determination of underivatized clonazepam in plasma, using nitrazepam as an internal standard (25.0 ng/ml). The mean recovery of clonazepam from plasma by the same extraction procedure was 75% (highest S.D. 5.9%; $n = 4$). The mean of three calibration graphs for clonazepam made on different occasions showed a highest S.D. value of 4.2%.

It appears that the present method permits the accurate and specific determination of underivatized nitrazepam and clonazepam in plasma in relatively low concentrations. The detection limit is about 1 ng nitrazepam or clonaze-

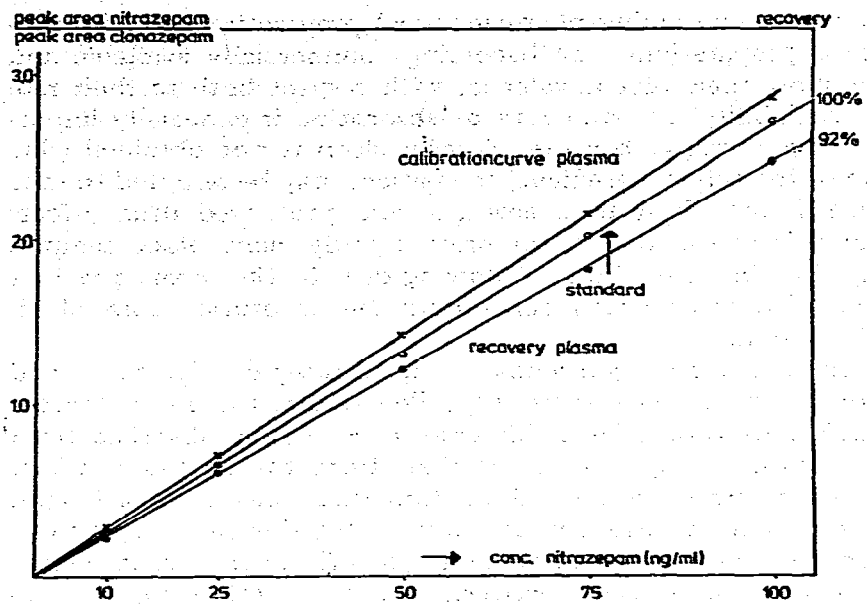

Fig. 5. Peak area ratio of nitrazepam to clonazepam (25.0 ng) as a function of known nitrazepam concentrations. The standard curve was obtained with stock solutions of the two drugs; the recovery line was obtained by extraction of nitrazepam from plasma, using clonazepam as external standard; the calibration graph was obtained by extraction of nitrazepam from plasma, using clonazepam as internal standard.

pam per ml of plasma. The use of the capillary OV-17 SCOT column appears to be a definite improvement in the analysis of these two benzodiazepines and many samples can be analyzed in short time. An addition advantage in using capillary columns is the low column bleeding which is particularly important when using electron-capture detection. A solid injection system is required in order to prevent deterioration of the column support, caused by organic solvents. Whether the present procedure is also suitable for monitoring nitrazepam or clonazepam in clinical situations requires further investigation, especially with reference to interference by co-administered medications. So far, interference by oxazepam, lorazepam, flurazepam, chlordiazepoxide, nordiazepam, diazepam, hydroxydiazepam and medazepam can be excluded. Preliminary studies on the metabolism of nitrazepam in humans have indicated that its major metabolites do not interfere with the assay of the parent compounds.

Pharmacokinetic and bioavailability studies in humans

The present assay procedure was primarily developed because of the need for more information concerning the pharmacokinetics and disposition of the extensively used hypnotic drug nitrazepam in humans following therapeutic dosage. So far, only the investigations by Rieder [9] have yielded reliable data on plasma elimination half-lives of nitrazepam in man. Apart from drug disposition data, there is a growing need for bioavailability deter-

minations of nitrazepam-containing pharmaceutical preparations. An increasing number of these preparations are becoming commercially available and it is important to show their bioequivalence, with respect both to their rate and extent of bioavailability. A rapid rate of absorption is expecially important in hypnotic drug therapy, because if early sleep is not obtained (due to slow absorption of the active ingredient) the patient may be tempted to take a second dose, which may lead to overdosage and prolonged drug effects [24, 25]. It is for this reason that in the present study many blood samples were taken during the first three hours following drug intake. From previous studies [9, 13] detailed information concerning the absorption rate of nitrazepam cannot be obtained.

Two tablet preparations, each containing 5 mg nitrazepam (Mogadon and Sameko), were compared in a cross-over way. The results for one volunteer are shown in Fig. 6. Nitrazepam is in this case more rapidly absorbed from the Sameko preparation (t_{max} = 45 min) than from the Mogadon tablet ($t_{max.}$ = 2 h). After termination of absorption there was an initial rapid decline of the plasma concentration, which is probably due primarily to distribution of the drug in the tissues. Subsequently, a definite increase in plasma concentration occurred in most cases between 4 and 7 h following drug administration. This may be caused by a redistribution process initiated by intercurrent food intake, as was recently shown to occur also for diazepam [26, 27].

Later the plasma concentration time course followed a monoexponential decay (Fig. 6), from which elimination half-lives could be calculated. The relevant pharmacokinetic parameters are summarized in Table I. The areas under the curve were normalized for the differences in elimination half-life

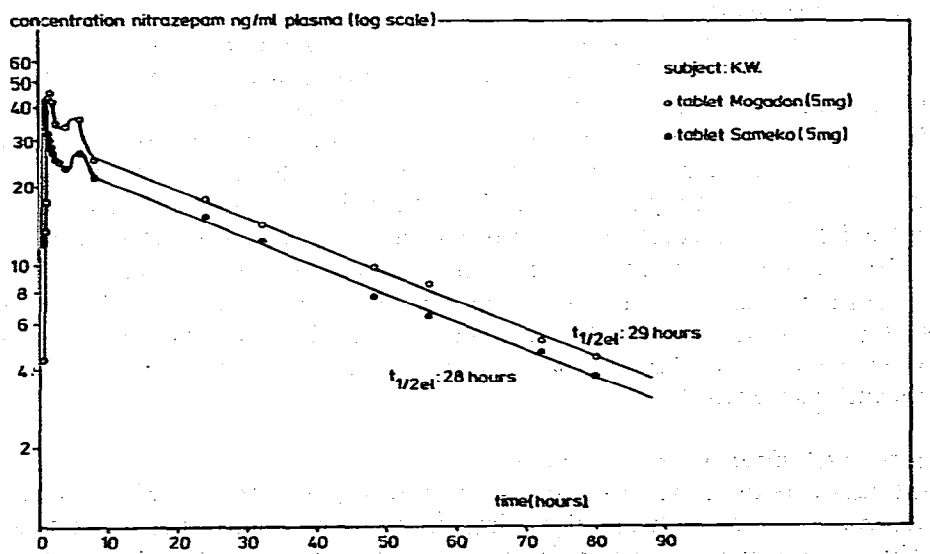

Fig. 6. Plasma concentration curves on semi-logarithmic scale of nitrazepam in a healthy volunteer following administration of a Mogadon tablet and of a Sameko tablet. The various pharmacokinetic parameters for this volunteer are given in Table I.

in the same individual and also for the undetermined area until infinity [28]. The elimination half-life of nitrazepam varied between 13 h and 34 h, with a mean value of 27 h, which result is in good agreement with that reported by Rieder [9]. There was less intra- than intersubject variability in elimination half-life (Table I). The average peak level times ($t_{max.}$) were 88 and 38 min for the Mogadon and Sameko tablets respectively. However, these values appeared not to be statistically significantly different (paired t test). With respect to the extent of bioavailability there is no important difference (despite substantial intersubject variation) between the two preparations.

TABLE I

VARIOUS PHARMACOKINETIC PARAMETERS

Elimination half-lives ($t_{½el.}$), peak level times ($t_{max.}$), areas under the plasma concentration curves (AUC), and relative bioavailability (%) of nitrazepam following the administration of Mogadon tablets (Mo) and Sameko tablets (Sa), each containing 5 mg nitrazepam.

Subject	$t_{½el.}$(h)		$t_{max.}$(min)		AUC(mg·h·l^{-1})		$\frac{AUC(Sa)}{AUC(Mo)} \times 100\%$
	Mo	Sa	Mo	Sa	Mo	Sa	
K.W.	28	28	120	45	1361	1101	81
L.B.	32	34	45	45	1301	1613	124
J.L.	32	33	30	30	909	1004	110
S.V.	28	28	30	45	1006	969	96
B.R.	26	25	60	45	815	754	92
P.B.	34	25	240	30	1318	1115	85
G.B.	18	13	90	30	972	566	58
mean values	28	27	88	38	1097	1017	92

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