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SENSITIVE DETERMINATION OF AMBENONIUM CHLORIDE IN SERUM FROM PATIENTS WITH MYASTHENIA GRAVIS USING ION-EXCHANGE RESIN EXTRACTION AND REVERSED-PHASE ION-PAIR CHROMATOGRAPHY

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SUMMARY

An effective and selective procedure for the extraction of ambenonium chloride (AMBC) from serum using a weak cation-exchange extraction cartridge has been developed The solid-phase extraction procedure permitted the extraction of AMBC from serum without adhesion to materials such as the containers A 200-μl volume of the eluate could be directly injected on to a reversed-phase ion-pair high-performance liquid chromatographic column The recovery was in the range 97–100% The limit of detection for AMBC was 0 5 ng/ml in serum (signal-to-noise ratio = 3) The method was used to determine the serum concentration of AMBC in patients with myasthenia gravis The method would be useful for monitoring AMBC in serum in order to study its pharmacokinetic behaviour in patients under oral administration therapy

INTRODUCTION

Cholinesterase inhibitors, which are quaternary compounds (Fig. 1), have been used in the symptomatic treatment of patients with myasthenia gravis (MG) Edrophonium chloride is only used as a diagnostic reagent owing to its rapid hydrolysis in the body. Ambenonium chloride (AMBC) and pyridostig-

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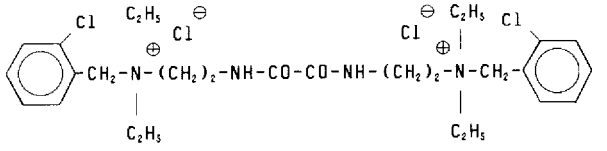
mine bromide (PDS) are extensively used for the treatment of MG, as they have longer pharmacological effects [1]

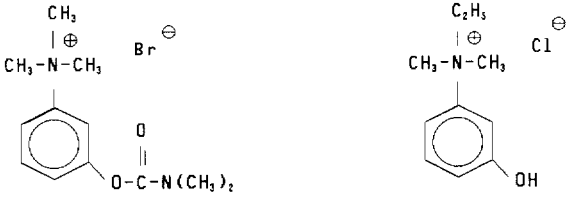
Recently, pharmacokinetic and pharmacodynamic studies on PDS have been reported [2,3]; however, there have been no reports on AMBC owing to the lack of a sensitive and facile method for its determination in serum from patients.

Only two methods have so far been reported for the determination of AMBC in biological specimens [4,5]. These were based on high-performance liquid chromatography (HPLC), involving liquid–liquid extraction of AMBC from the native samples followed by reversed-phase separation and UV detection. However, these methods did not consider the effect of the considerable adhesion of AMBC to materials such as containers. Moreover, concentrations of AMBC lower than 10 ng/ml in serum from patients were not considered. Hence there is still a need for a method for monitoring AMBC in serum from patients under the drug therapy.

The aim of this work was to develop an effective and selective procedure for the extraction of AMBC from patients' serum using a solid-phase extraction cartridge. In the method presented, adhesion of AMBC was avoidable because siliconized materials could be used in the whole process of extraction of AMBC from serum. A 200-μl volume of eluate from the cartridge could be injected into the HPLC system and the limit of detection for AMBC was 0 5 ng/ml in serum



(A)



(B)

(C)

Fig 1 Structures of cholinesterase inhibitors (A) Ambenonium chloride, (B) pyridostigmine bromide, (C) edrophonium chloride

The extraction and determination method was used to monitor the serum concentration of AMBC after oral administration of this drug to patients with MG.

EXPERIMENTAL

Reagents

AMBC and timepidium bromide were kindly supplied by Nippon Shoji Kaisha (Kyoto, Japan) and Tanabe Pharmaceutical (Tokyo, Japan), respectively Acetonitrile was of HPLC grade and all other chemicals were of analytical-reagent grade (Wako, Osaka, Japan). Distilled water was passed through a Milli-Q™ system (Millipore, Bedford, MA, U.S.A.) Drug-free serum was purchased from Bio-Rad Labs. (Anaheim, CA, U.S.A) A Bond-Elut CBA® extraction cartridge, which was packed with a weak cation-exchange resin, was obtained from Analytichem International (Harbor City, CA, U.S.A.).

Solutions

Sodium phosphate buffer (0.2 *M*, pH 6.0) was prepared in the usual manner and was used to equilibrate the Bond-Elut CBA cartridge, to dilute serum samples and to wash the cartridge after applying the sample. Internal standard (I S.) solution (0.2 μg/ml) was prepared by dissolving timepidium bromide in water. The eluent was acetonitrile–2 *M* lithium perchlorate (1 1, v/v), and was used to elute AMBC from the cartridge.

AMBC standard solutions (5–400 ng/ml) were prepared in water and stored in Teflon containers at 5°C AMBC was very stable under these conditions and the solutions were usable for more than three months However, caution should be exercised in the sample preparation of AMBC because of its considerable adhesion to various materials. All the materials used, which include glass and polypropylene containers, reservoirs and pipette tips, should be siliconized Standard sera was obtained by dissolving the drug-free lyophilized serum in the standard solutions of AMBC.

Standard sera were used to prepare a calibration graph for the drug at concentrations of 0, 2 5, 5.0, 10, 25 and 50 ng/ml.

HPLC system

The HPLC system consisted of a Model 510 pump, a Model 490 programmable multi-wavelength detector operating at 214 nm, a Model 712 automated sample injector (Waters Assoc., Milford, MA, U S.A.) and Shimadzu (Kyoto, Japan) Chromatopac C-R1A data processor A TSK-gel ODS-80T_M reversed-phase column (150 mm × 4 6 mm I D , particle size 5 μm, Tosoh, Tokyo, Japan) was used The mobile phase was acetonitrile–ammonium chloride buffer (0.1 *M*, pH 3 5) containing 25 m*M* lithium perchlorate (3 7, v/v). The flow-rate was 1 ml/min and the column temperature was 25°C Peak-height ratios of the drug to the internal standard were used for the quantification

Serum sample

Blood samples were obtained from patients undergoing AMBC therapy as part of the current treatment protocol being utilized in the Department of Neurology, Neurological Institute, Kyushu University Hospital (Fukuoka, Japan). The specimens were collected in siliconized plain plasma glass tubes and centrifuged at 1500 *g* for 10 min. The serum was stored at −20 °C in siliconized polypropylene sample cups; the drug concentration did not change under these conditions for two weeks. Other investigators have reported similar stability of AMBC in plasma [5]

Extraction procedure

The Bond-Elut CBA cartridge was washed successively with 1 ml of the solution solvent, 1 ml of water (twice), 1 ml of acetonitrile, 1 ml of water (twice), 1 ml of 0 1 *M* hydrochloric acid (twice), 1 ml of water (three times) and 1 ml of phosphate buffer (twice) before use Serum (1 0 ml) was spiked with the I.S solution (0.5 ml) and diluted with 40 ml of phosphate buffer, and the mixture was passed through the Bond-Elut CBA cartridge at a rate of ca 2.5 ml/min by using a Vac-Elut SPS 24 system (Analytichem International) The cartridge was washed successively with 5 ml of phosphate buffer (three times), 1 ml of water (three times), 0.5 ml of 50% (v/v) acetonitrile and 1 ml of water. AMBC was eluted with 0.25 ml of the eluent and 200 μl of the eluate were injected into the HPLC system.

RESULTS AND DISCUSSION

The adhesional characteristics of AMBC were investigated using aqueous solutions and serum spiked with the drug In glass tubes, the proportions of AMBC (10 ng/ml) remaining in the solution and in serum after 1 h were about 60 and 90%, respectively After 24 h, these levels were less than 25 and 50%, respectively. The amount of AMBC (10 ng/ml) remaining in serum when stored in a polypropylene tube for 24 h was about 60% In a siliconized glass tube or a PTFE container, the amount of AMBC (10 ng/ml) in aqueous solution or in the eluent remained unchanged for more than 24 h The extent of adhesion may be variable under the different conditions. Owing to the considerable adhesion to various materials, all glass containers, pipette tips and reservoirs should be siliconized before use or PTFE containers should be used.

The conditions for ion-pair reversed-phase HPLC used in the method were similar to those reported previously [4]. Fig. 2 shows typical chromatograms obtained by the method with drug-free normal human serum spiked with AMBC (5 ng/ml) and the blank. Fig 3 shows the chromatograms obtained with patients' sera before and after oral administration of AMBC. AMBC and timepidium bromide had retention times of ca. 8.5 and 21 min, respectively. We were not able to identify the metabolites on the chromatogram.

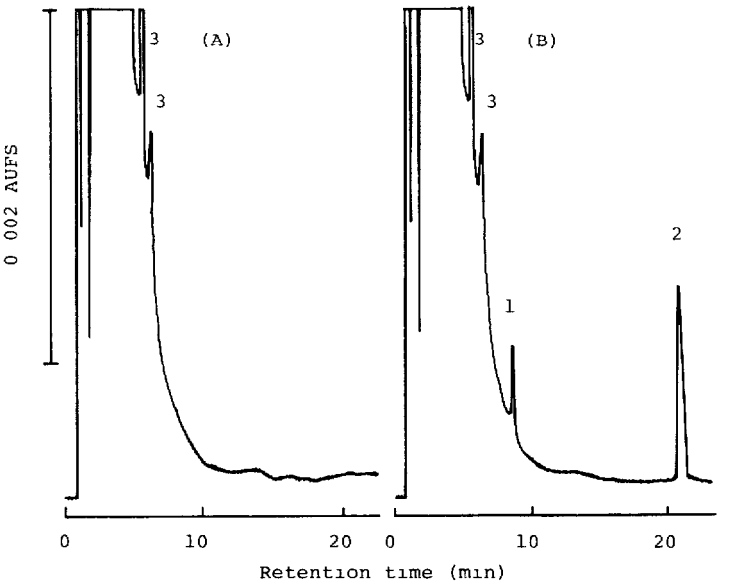


Fig 2 Chromatograms obtained with drug-free normal human serum (A) and with that spiked with ambenonium chloride (5 ng/ml) and timepidium bromide as an internal standard (B) Peaks 1 = ambenonium chloride, 2 = timepidium bromide (I S), 3 = unknown

The peak of AMBC on the chromatogram showed a shoulder owing to the presence of endogenous compounds. Therefore, the Shimadzu Chromatopac C-R1A was set to draw baselines between valleys where the peaks did not meet the baseline. The data processor integrated the peak height with good precision (Table I). A calibration graph was prepared in the concentration range 0–50 ng/ml, which covers the levels of AMBC usually exhibited in serum from patients. The peak height was linearly related to the drug concentration in this range ($y=0.07152x+0.0009$, $r=0.9991$, duplicate determinations) The minimum concentration of AMBC detectable was 0.5 ng/ml in serum (signal-to-noise ratio = 3); this sensitivity was improved twenty-fold over that in the previous method [4].

The within-day precision of the method was investigated by using standard sera (2.5, 5 and 10 ng/ml). The relative standard deviation did not exceed 5% (Table I). This precision was satisfactory for monitoring the serum concentrations in patients under AMBC therapy.

The test of the recovery of AMBC from standard sera using the extraction procedure with the ion-exchange cartridge was carried out by comparing the peak heights obtained from standard solutions of AMBC. The recovery was dependent on volume, pH and salt concentration of the phosphate buffer used to dilute the serum and to wash the cartridge after applying serum samples The use of a small volume of the phosphate buffer to dilute serum reduced the

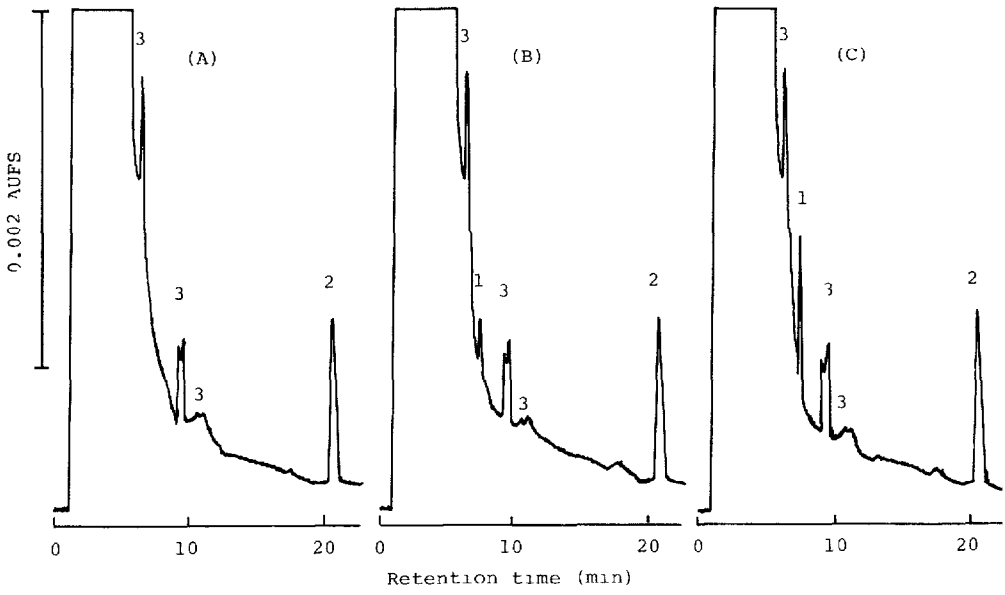


Fig 3 Chromatograms obtained from sera from patients receiving ambenonium chloride orally (A) Just before administration, (B) 30 min after administration, (C) 60 min after administration Peaks 1 = ambenonium chloride, 2 = timepidium bromide (I S), 3 = unknown

TABLE I

REPRODUCIBILITY OF THE DETERMINATION OF AMBENONIUM IN SERUM SAMPLES

Concentration added (ng/ml)	Relative standard deviation ($n=9$) (%)
2 5	4 9
5 0	4 1
10 0	3 2

recovery, a 50% recovery was observed when serum was diluted ten-fold and complete recovery (>97%) was achieved with a forty-fold dilution At lower pH of the phosphate buffer the recovery decreased; the maximum and stable recovery was attained at pH 6.0 or higher and hence pH 6 0 was adopted in the procedure. A moderate salt concentration in the phosphate buffer was necessary to remove serum protein from the resin When 0.05 *M* phosphate buffer was used, the cartridge could not be freed from interfering compounds and the eluate that contained organic solvent became opaque. Even when 0.1 *M* phosphate buffer (5 ml) was passed through the cartridge three times, the recovery was low and irregular; 0.2 *M* was selected as the optimum concentration. The

cartridge should be further washed with 50% (v/v) acetonitrile to remove the endogenous compounds.

The ionic strength and the organic solvent concentration in the eluent solvent affected the elution of AMBC from the resin. Generally, a sample charged to the Bond-Elut CBA cartridge is eluted with two aliquots of half the resin volume each of buffer of either higher ionic strength (>0.1) or lower pH, and the addition of an organic modifier such as methanol to the elution buffer may improve the recovery A highly ionized compound such as potassium chloride caused a decrease in the peak height of AMBC at concentrations greater than 0.05 *M* in the eluent, the compound may be considered to interfere with the formation of the ion pair with perchlorate and AMBC However, lithium perchlorate added as an ionic compound to the eluent, which was also a component of the mobile phase, afforded a good result; almost a maximum and constant recovery of AMBC from the resin was achieved at lithium perchlorate concentrations greater than 1.5 *M* (Fig. 4); 2 *M* was adopted in the procedure The optimum acetonitrile concentration in the eluent was 50% (v/v). Lower or higher concentrations caused a decrease in the recovery and also a broadened

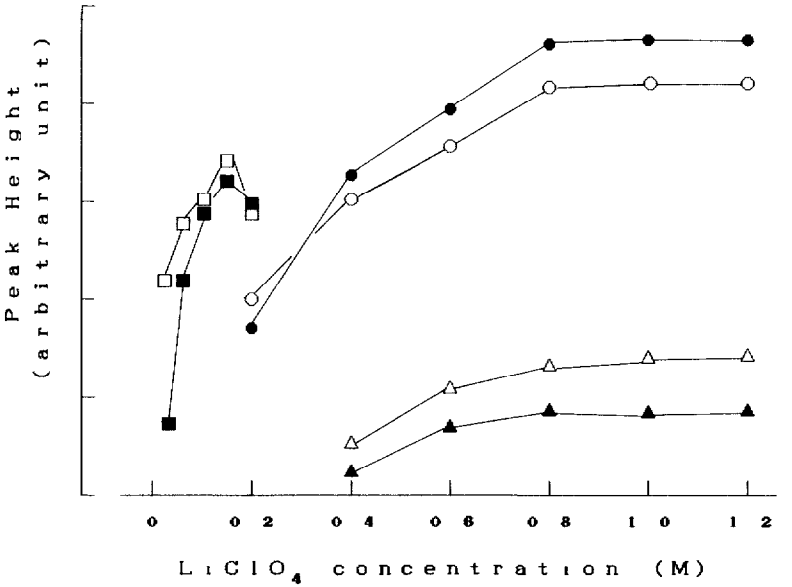


Fig 4 Effect of lithium perchlorate and acetonitrile concentration in the eluent Each value is the average from triplicate analyses of standard serum (10 ng/ml) with addition of an internal standard (100 ng) The eluents were prepared by mixing lithium perchlorate solutions and pure or aqueous acetonitrile, and the lithium perchlorate and acetonitrile concentrations were adjusted to those indicated in the figure (■) Ambenonium chloride and (□) timepidium bromide (I S)-90% (v/v) acetonitrile, (●) ambenonium chloride and (○) timepidium bromide (I S)-50% (v/v) acetonitrile, (▲) ambenonium chloride and (△) timepidium bromide (I S)-30% (v/v) acetonitrile

TABLE II

EXTRACTION RECOVERIES OF AMBENONIUM FROM SERUM SAMPLES

Concentration added (ng/ml)	Recovery (mean ± S D, $n=6$) (%)
2 5	97 7 ± 4 4
10 0	100 0 ± 2 9

peak Under the above conditions, a maximum and constant recovery (97–100%) was obtained (Table II).

We tested the addition of several quaternary ammonium compounds to serum (1 μg/ml) using the proposed method: methylbenactyzium bromide, oxapium iodide, domiphen bromide, propanthline bromide, clocapramine hydrochloride, benzethonium chloride, benzalkonium chloride, neostigmine bromide, distigmine bromide and pralidoxime iodide Of these compounds, only the peak of methylbenactyzium bromide was near that of AMBC The retention time was 12 min, so it did not interfere with the determination of AMBC Drugs given concomitantly to patients with MG, such as prednisolone and atropine sulphate, did not interfere

Several quaternary ammonium compounds that had ester structures were examined as possible internal standards, but they are not recommended because of their instability in serum or aqueous solutions Timepidium bromide was very stable under the conditions adopted and the recovery was good, so it was selected as the internal standard.

We determined AMBC concentrations in patients' serum by the proposed method after oral administration of the drug. Serum concentration–time profiles were obtained in two instances (Fig 5). Patient A was given an oral dose of 10 mg of AMBC and patient B was given two doses of 40 and 30 mg in the sampling period. Patients A and B received previous doses of 10 mg at 4 h and 5 mg at 14.5 h before the beginning of sampling, respectively The profile of patient A showed a peak serum concentration (C_{max}) immediately after oral administration That in patient B also showed a C_{max} after the first dose, but not after the second The reason for the absence of a C_{max} was not elucidated The time to reach C_{max} was almost the same in both instances (1–1.5 h) C_{max} normalized with a dose was different in the two instances, being 0 84 ng/ml·mg in patient A and 0 20 ng/ml·mg in patient B Only one report of human serum AMBC concentrations has been published· Bloch et al. [4] reported C_{max} values of about 20 and 40 ng/ml with oral doses of 5 and 10 mg, respectively However, the concentration of AMBC in serum from patients was not as high in our study

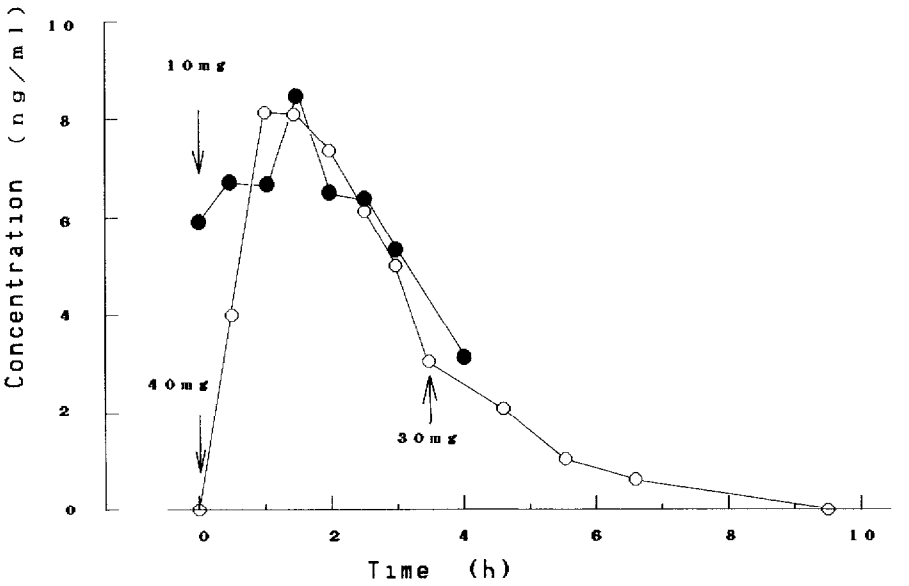


Fig 5 Serum ambenonium concentration-time profiles in two patients with myasthenia gravis under multiple oral therapy Patient A (●) received 10 mg of AMBC Patient B (○) received 40 and 30 mg at the interval shown Each value is the average of duplicate determinations Patient A (C K), female, age 33 years, height 149 cm, weight 65 kg, daily dose 50 mg, patient B (N T), female, age 42 years, height 158 cm, weight 55 kg, daily dose 80 mg

The correlation between dose and serum concentration was investigated in patients who had received AMBC under a diet or under fasting (Fig. 6) AMBC concentration was determined in serum collected 3 h after dosing, and dose was normalized with body weight We could not obtain a clear correlation in either group because of a large inter-individual variability of the serum concentrations The effect of diet before administration was investigated when the serum AMBC concentration was normalized with dose versus body weight The mean value ± standard deviation was 0.126 ± 0 164 ng/mg·kg ($n=10$) under a diet and 0 295 ± 0 214 ng/mg·kg ($n=17$) under fasting In view of the low bioavailability of reversible cholinesterase inhibitors, the serum concentration of AMBC was affected by various factors, involving ingestion of food and comedications [1] These effects remain to be confirmed by more studies

Pharmacokinetic studies may be advanced by the more intensive determination of serum concentrations of AMBC, and the proposed method should be useful for such studies

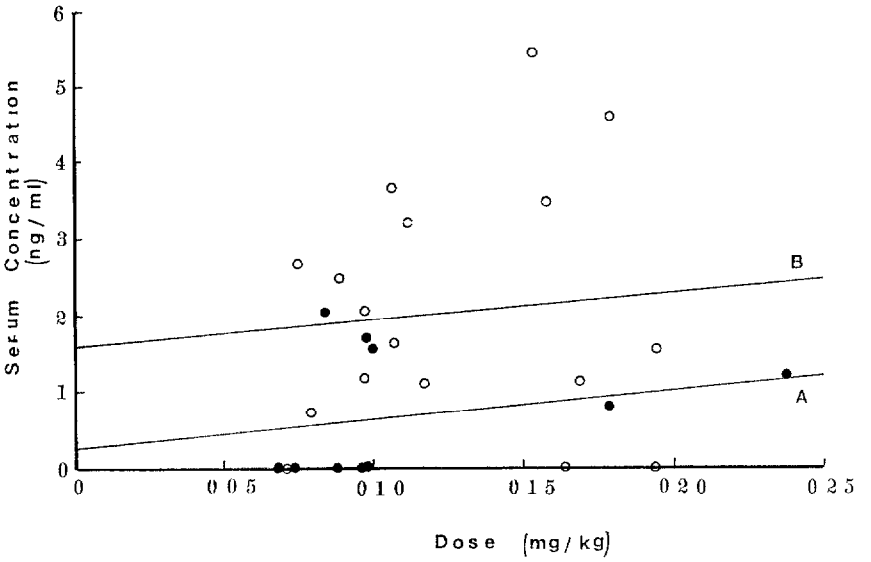


Fig 6 Relationship between serum ambenonium chloride concentration and normalized dose with body weight (mg/kg) The blood was collected 3 h after oral administration of ambenonium chloride under a diet (●) or fasting (○) Regression line A, under a diet ($y=3\ 7632x+0\ 3035$, $r=0\ 2407$, $n=10$), regression line B, under fasting ($y=3\ 5880x+1\ 5846$, $r=0\ 0950$, $n=17$) Patients under diet number, ten (five male, five female), age, 45 9 ± 14 0 years, body weight, 55 5 ± 8 4 kg, study dose, 6 0 ± 2 1 mg, serum concentration, 0 73 ± 0 83 ng/ml Patients under fasting number, seventeen (eight male, nine female), age, 42 7 ± 14 0 years, body weight, 58 4 ± 11 2 kg, study dose, 7 4 ± 2 6 mg, serum concentration, 2 04 ± 1 61 ng/ml

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