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Determination of semicarbazide-sensitive amine oxidase activity in human plasma by high-performance liquid chromatography with fluorimetric detection

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Abstract

We report here a simple and sensitive method for the measurement of semicarbazide-sensitive amine oxidase (SSAO) activity in human plasma. Benzaldehyde, generated during a 1-h incubation of plasma with benzylamine, is derivatized with the specific aldehyde reagent dimedone after prior deproteinization. Quantitation of the derivatization product is done by automated injection onto an isocratic high-performance liquid chromatographic system with fluorimetric detection. The assay shows good linearity and reproducibility (intra-assay C.V. 7%). Detection limit is 25 mU/l (= pmol/ml/min). In 51 healthy controls (age 49 ± 13 yr, 20 males) the measured SSAO activity was 352 ± 102 mU/l (mean ± S.D.). A large number of samples (70–80) can easily be processed in one day by one technician.

1. Introduction

Human plasma contains an amine oxidase which is known under various names, such as benzylamine oxidase, clorgyline-resistant amine oxidase and semicarbazide-sensitive amine oxidase (SSAO; EC 1.4.3.6) [1]. SSAO is the common name for a group of heterogeneous enzymes which vary in characteristics depending on the source of the SSAO (different animals, different tissues) [2–7]. All SSAO enzymes differ from the monoamine oxidases MAO-A and MAO-B in their inhibition pattern: they are insensitive to MAO inhibitors like clorgyline, pargyline and deprenyl, but sensitive to semicarbazide and other hydrazines. SSAO is a copper-containing enzyme; pyridoxal phosphate has long been thought to be a cofactor, but recent reports suggest that pyrroloquinoline quinone or 6-hydroxydopa is the real cofactor [8,9]. The natural substrate of SSAO is not known, but benzylamine, tyramine and tryptamine are all good substrates. Other amines reported to be oxidized by SSAO include dopamine, polyamines, methylamine and allylamine. Conversion of the latter to highly toxic acrolein has been suggested to be the cause of the cardiovascular toxicity of allylamine [10], while oxidative deamination of methylamine would

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lead to the formation of cytotoxic formaldehyde [6,11].

SSAO is present in many organs and tissues, but is especially associated with highly vascularized tissues, arteries and smooth muscle cells. Plasma SSAO has been reported to be increased in patients with diabetes mellitus [12,13], chronic congestive heart failure [12] and liver disease [14], while decreased concentrations were reported in severely burned patients [15], in patients with cancer [12,15], and in patients treated with corticosteroids [12].

Several methods have been described for the measurement of SSAO activity, mostly using benzylamine as the preferred substrate. These include spectrophotometric assays [1,16], radiochemical techniques [17–21], and methods based on measuring the hydrogen peroxide generated during the oxidative deamination [22,23]. Most of these methods are not well suited to routine measurements in large numbers of samples due to their complexity, cost, use of radioactive compounds or lack of sensitivity for measurement of the relatively low SSAO activity in human plasma.

We report here a simple, sensitive and reproducible method which allows the processing of a large number of samples. The method is based on the derivatization of benzaldehyde, generated during incubation of plasma with benzylamine, with the highly specific aldehyde reagent dimedone (5,5-dimethyl-1,3-cyclohexanedione [24]) and subsequent quantitation by isocratic high-performance liquid chromatography with fluorimetric detection.

2. Experimental

2.1. Reagents

Benzaldehyde, dichloromethane, trichloroacetic acid, sulphuric acid and 2-propanol were obtained from Merck (Darmstadt, Germany). Benzylamine, dimedone, pargyline, clorgyline, semicarbazide, hydralazine, benzyloxyamine, phenelzine, 6-hydroxydopa, β-aminopropionitrile and isoniazid were supplied by Sigma (St.Louis, MO, USA), and acetonitrile by Baker (Deventer, Netherlands).

(E) - 2 - (3′,4′-Dimethoxyphenyl) - 3 - fluoroallylamine (MDL-72,145A, I) and (E) - 2 - (4 - fluorophenethyl) - 3 - fluoroallylamine (MDL-72,974A, II) were kindly provided by the Marion Merrel Dow Research Institute (Strasbourg, France); carbidopa and benserazide were gifts from Merck Sharp and Dohme (Haarlem, Netherlands) and Hoffman-la Roche (Basle, Switzerland), respectively.

2.2. Instrumentation

The HPLC system used consisted of a Kratos SF-400 pump (Kratos, Ramsey, NJ, USA), a Bio-Rad AS 100 autosampler (Hercules, CA, USA), a Merck-Hitachi D2500 integrator (Tokyo, Japan) and a Shimadzu RFR 535 spectrofluorimeter (Kyoto, Japan). Separations were performed on 3-μm Spherisorb ODS2 (150 × 4.1 mm I.D.) cartridges (Phase Separations, Deeside, UK) protected by reversed-phase guard cartridges.

2.3. Solutions

All water used was purified by a Milli-Q system (Millipore, Bedford, MA, USA) and subsequently washed with dichloromethane.

Benzaldehyde solution (BAL) was prepared by dissolving 25 μl of benzaldehyde in 10 ml of 2-propanol–water 1:1 (v/v). A working solution (0.049 m*M*) was prepared by 10-fold dilution with the same mixture, followed by a further 50-fold dilution with bidistilled water, aliquotted in polyethylene tubes and stored at −80°C.

A stock solution of benzylamine was daily freshly prepared by dissolving 75 mg of benzylamine hydrochloride in 10 ml of 0.01 *M* hydrochloric acid. Five milliliters of this stock solution were diluted with an equal volume of 0.01 *M* hydrochloric acid to obtain the working solution with a final concentration 26.11 m*M*.

Sulphuric acid solution was prepared by adding 50 ml of concentrated sulphuric acid to 50 ml of water, and gentle refluxing in a round-bottom flask on an oil bath to remove aldehyde im-

purities. After 2 h the solution was cooled and stored in a refrigerator.

Dimedone solution was prepared by dissolving 2.10 g of dimedone in 17.5 ml of 2-propanol and 75 ml of water, adding 60 g of ammonium acetate, and water to a final volume of 100 ml. The solution was gently stirred overnight with 50 ml of dichloromethane. After discarding the dichloromethane, the solution was washed twice with 25 ml of dichloromethane, once with 25 ml of acetonitrile, again twice with 25 ml of dichloromethane, and stored in a refrigerator. The pH of the solution should be 6.5 ± 0.5. An alternative method for purification of the dimedone solution is to pass the solution through a Sep-Pak C_{18} column (Waters, Milford, MA, USA) activated with respectively 5 ml of water and 5 ml of methanol.

2.4. Assay

To an Eppendorf vial were added 350 μl of sodium phosphate buffer (0.1 *M*, pH 7.8) and 50 μl of a solution of clorgyline in 0.01 *M* hydrochloric acid (9.07 m*M*). After the addition of 50 μl of plasma, the mixture was preincubated in a shaking water bath at 37°C for 30 min, and the reaction was started by the addition of 50 μl of the benzylamine working solution. After exactly 60 min incubation in the shaking water bath at 37°C the reaction was terminated by the addition of 50 μl of 40% trichloroacetic acid. The vial was cooled in an ice-bath and then centrifuged in an Eppendorf centrifuge (10 000 *g*) for 3 min.

Blanks and standards were prepared by pipetting into an Eppendorf vial 300 μl of the phosphate buffer, 50 μl of the clorgyline solution, 50 μl of plasma pool, 25 μl of 18 m*M* MDL-I in 0.01 *M* hydrochloric acid, and 50 μl of water or benzaldehyde working solution. This mixture was treated exactly as described for plasma samples, except that incubation is started with 25 μl of the stock solution of benzylamine.

2.5. Derivatization

To an Eppendorf vial were added 400 μl of the supernatant resulting from the assay, 50 μl of 9 *M* sulphuric acid, and 200 μl of dimedone solution. A small hole was pierced in the cover of the vial, and the mixture was incubated at 95°C in an Eppendorf thermostat. After 45 min the vial was cooled in an ice-bath, vortex-mixed, and introduced into the autosampler.

2.6. Chromatography

Five microliters were injected onto the chromatographic system. The mobile phase was water–acetonitrile (50:50, v/v) at a flow-rate of 1.0 ml/min. The spectrofluorimeter was operated at 386 nm (excitation) and 451 nm (emission).

2.7. Calculation

The SSAO activity, expressed as pmol benzaldehyde formed per ml plasma per minute (= mU/l), was calculated by dividing the measured peak area of a sample by the average peak area of the standard (both corrected for the average blank value) and multiplying by the absolute amount of BAL standard in the incubation mixture times 20/60.

All experiments were performed at least in duplicate. For the sake of clarity standard deviations (always $< 10\%$) have not been included in the figures.

3. Results and discussion

3.1. Derivatization with dimedone

In preliminary experiments with benzaldehyde (BAL) solutions in 0.01 *M* hydrochloric acid the optimal conditions for derivatization were found to be similar to those described by Mopper et al. [24]. At 95°C the reaction proceeded smoothly, although in our hands maximal and stable fluorescence was reached after 30–60 min (Fig. 1). We have therefore used a reaction time of 45 min. At lower temperatures, the reaction proceeded much slower, and did not reach the same peak area as at 95°C: 63 and 78% after 1 and 2 h respectively at 56°C, and only 16 and

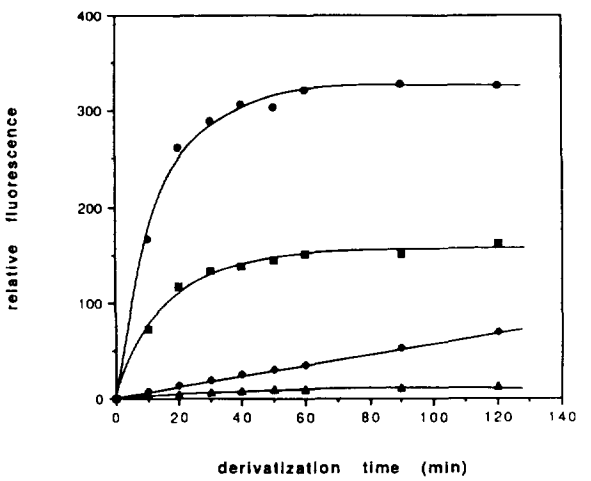

Fig. 1. Time and temperature dependence of the derivatization reaction. Benzaldehyde (2.47 nmol) at 95° (■), 56° (◆) and 37° (▲) C. Benzaldehyde (4.95 nmol) at 95°C (●).

20% after 1 and 2 h respectively at 37°C (Fig. 1). The pressure build-up at 95°C inside the vials sometimes caused caps to flew off, and therefore we pierced a small hole in the caps to prevent this from happening. The fluorescence was maximal when the spectrofluorimeter was set to 386 nm for excitation and to 451 nm for emission. BAL eluted well-separated from other peaks with a 50:50 water–acetonitrile mixture (Fig. 2). The height of the front peaks, blank value and other interferences were greatly reduced by the clean-up procedures with water and sulphuric acid as described in Experimental. Even with extended clean-up a small peak was still visible at the retention time of BAL. This blank value was determined in each assay and subtracted from sample and standard peaks.

When BAL dissolved in plasma was derivatized, the peak area was less than half of the area

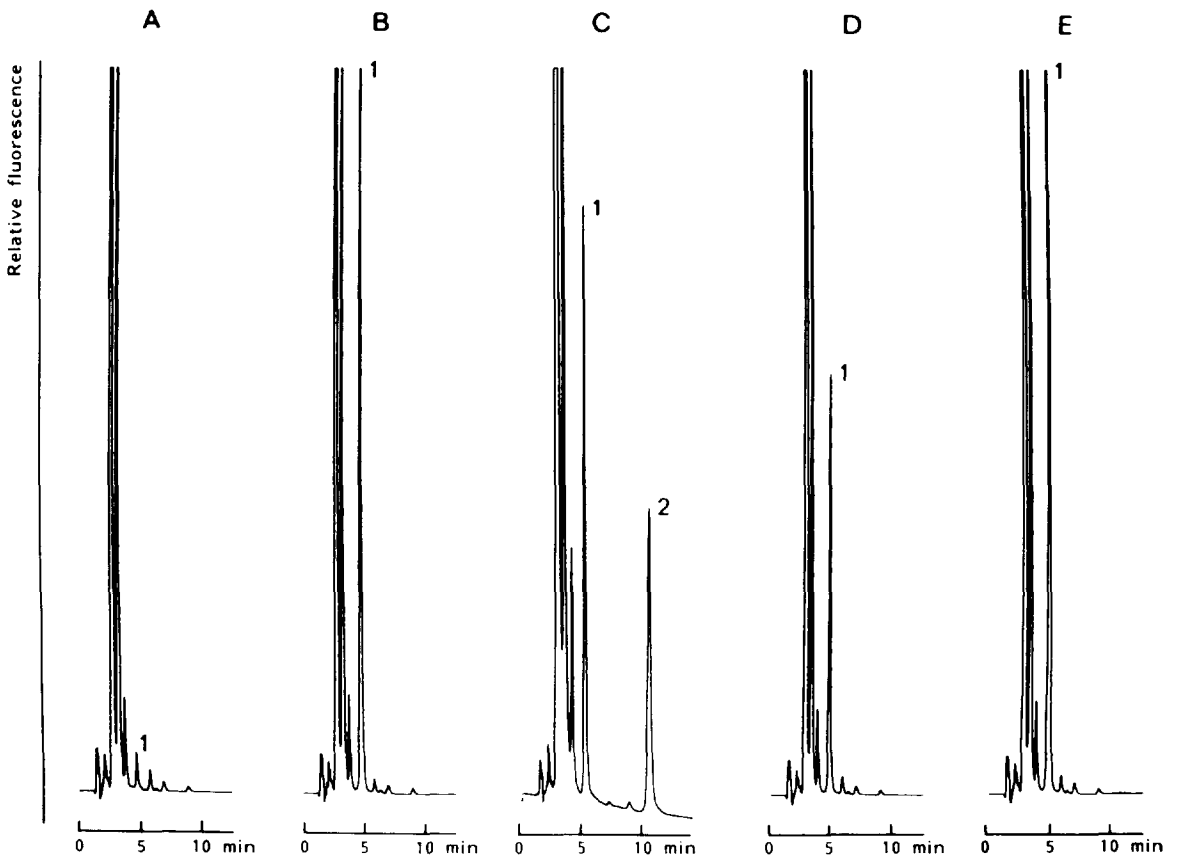

Fig. 2. Chromatograms. (A) Blank, (B) standard benzaldehyde (2.47 nmol), (C) standard benzaldehyde and hexanal (2.47 nmol), (D) and (E) plasma samples (400 and 1250 mU/l). Peaks: 1 = benzaldehyde 2 = hexanal.

obtained when the same amount of BAL was dissolved in hydrochloric acid. Apparently the protein matrix inhibited the derivatization reaction. The inhibition was largely overcome when plasma was first deproteinized by 3 *M* trichloroacetic acid. In the supernatant obtained after centrifugation the derivatization reaction proceeded quite well, although still slightly less than in hydrochloric acid (see section 3.7). Addition of sulphuric acid was still necessary: without addition derivatization did not occur. Optimal derivatization occurred at a pH of the dimedone solution of 6–9, with a resulting pH of the reaction mixture of 4.2–5.1.

The fluorescent derivatization product was stable for at least 72 h in the autosampler (cooled to 4°C).

A compound analogous to dimedone, 1,3-cyclohexanedione, has been reported to be an alternative reagent with some advantages over dimedone [25]. Experiments with BAL at 95°C showed however that the peak areas obtained with 1,3-cyclohexanedione were about 30% lower than those obtained with dimedone.

3.2. Pre-incubation

To prevent possible participation of plasma MAO in the conversion of the substrate benzylamine (BAM) to BAL, the reaction mixture (350 μl of phosphate buffer pH 7.8 and 50 μl of plasma) was first pre-incubated with 50 μl of a clorgyline solution (final concentration 0.9 m*M*) for 30 min. Under these circumstances any plasma MAO activity is completely blocked [26]. Higher concentrations of clorgyline caused a cloudiness in the reaction mixture as noted before [6]. In practice, the addition of clorgyline had no effect on the measured rate of formation of BAL: in 20 plasma samples measured with and without clorgyline results were similar (mean ± S.D. was 499 ± 216 and 506 ± 208 mU/l, respectively).

3.3. Incubation

After preincubation the reaction was started by the addition of 50 μl of BAM. Varying the BAM concentration showed that the maximal reaction rate was reached with a substrate concentration of 2.6 m*M* in the incubation mixture (Fig. 3). The production of BAL proceeded linearly for up to 2 h; as the amount of BAL formed after 1 h was large enough to be easily measurable, we choose a 1-h period for the incubation step. Optimal values were obtained with a pH of the phosphate buffer between 7.4 and 8.2. BAL was stable under the incubation conditions: (1) when BAL (and not BAM) was added to plasma and derivatized directly and after prior incubation, the same results were obtained, and (2) recovery of BAL added to 9 plasma samples prior to incubation was 96.6 ± 5.4% (mean ± S.D.).

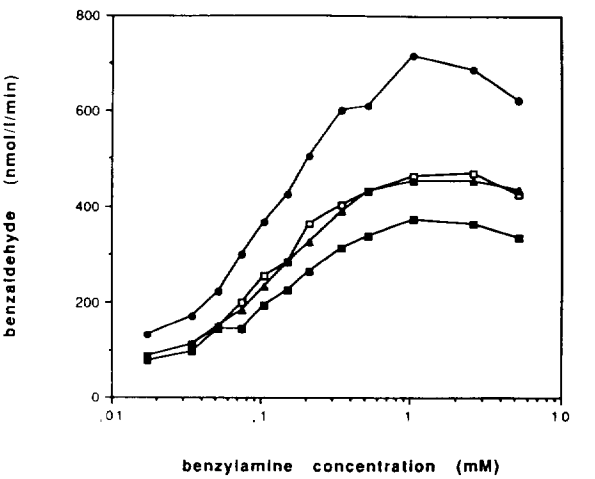

Fig. 3. Dependence of benzaldehyde formation on the benzylamine concentration in 4 different plasma samples. Assays were performed as described but with differing amounts of benzylamine in the incubation mixture.

3.4. Inhibitors

Several known inhibitors of SSAO were tested for effects on the generation of BAL as well on the derivatization reaction with dimedone. Benzyloxyamine, phenelzine, benserazide and β-aminopropionitrile (0.5 m*M*) did not hamper the derivatization of BAL, but gave strongly interfering peaks in the chromatogram. Semicarbazide, hydralazine, isoniazide, carbidopa, MDL-I and MDL-II (0.5 m*M*) inhibited SSAO,

without interfering in the derivatization of BAL or in the chromatogram. Estimated IC50 values were 10 μM for semicarbazide, 0.6 μM for hydralazine, 0.2 μM for carbidopa, 16 μM for MDL-I, 8 μM for isoniazide and 0.012 μM for MDL-II (Fig. 4). The interesting striking difference in IC50 between the two structurally related MDL compounds has recently also been noted by Yu et al. [27]. The MAO-B inhibitors selegeline and pargyline (1.1 mM) had no effect on the measured SSAO activity.

Mixing experiments of plasmas with high and with low SSAO activity gave results identical to the calculated results, indicating that no inhibitors and/or activators play a role in normal plasma measurements. In the presence of 6-hydroxydopa (up to 50 μM) measured SSAO activities were identical to those obtained without added 6-hydroxydopa.

3.5. Linearity

The derivatization reaction of BAL with dimedone gives linear fluorescence responses up to at least 400 nmol, which would correspond to an SSAO activity of 95 U/l. This is far in excess of the formation of BAL in human plasma, which proceeds linear for at least up to 2 h (Fig. 5). The highest activity in human plasma we have found so far is 2000 mU/l.

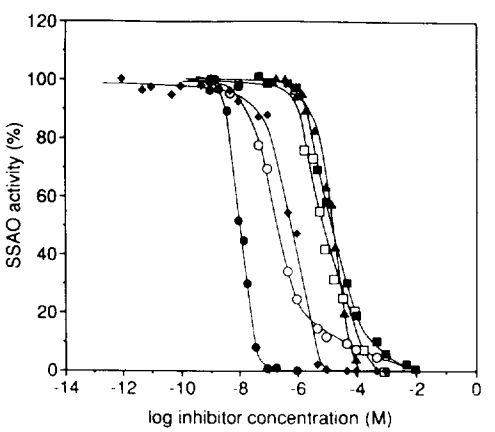

Fig. 4. Inhibition of SSAO activity by various compounds. (◆) Hydralazine, (□) isoniazide, (■) semicarbazide, (○) carbidopa, (●) MDL-II, (▲) MDL-I.

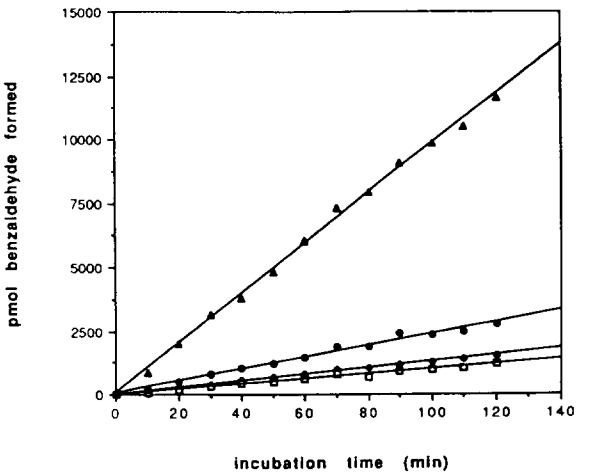

Fig. 5. Linearity of the assay with respect to incubation time in 4 different plasma samples (SSAO activity 194, 271, 475 and 2002 mU/l).

3.6. Detection limit

The detection limit of the assay is not limited by the sensitivity of the fluorimeter (which can still be increased by a factor 100), but by the blank value consistently obtained even after extensive clean-up of all solvents used. Taking an SSAO activity which produces a peak with twice the area of the blank as the detection limit for the described method for SSAO measurement, the detection limit is 25 mU/l. The lowest SSAO activity we have so far measured in human plasma is about four times higher.

3.7. Standardization

The peak area of the BAL derivative decreased with increasing amounts of plasma in the incubation mixture, despite the subsequent deproteinization step (Fig. 6). When the amount of plasma was kept constant by adding more plasma after the incubation, but before the deproteinization step, peak areas did not differ significantly. As it is thus important to have the same matrix for the reaction of the standards as for the samples, plasma was also used in the incubation mixtures for the standards, with MDL-I (0.9 mM) included to prevent formation of BAL. As

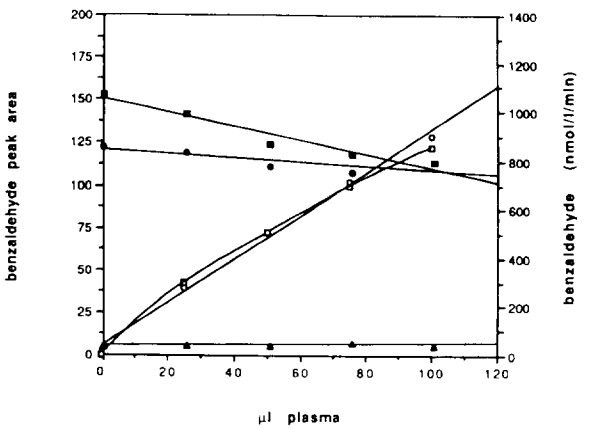

Fig. 6. Influence of varying amounts of plasma. (a) (left ordinate) Incubation of benzaldehyde (2.47 nmol) with varying amounts of plasma in the presence of MDL-I; volume was made equal with bidistilled water (■) or with plasma (●) after the incubation but before the derivatization. (b) (left ordinate) Incubation of bidistilled water with varying amounts of plasma (▲). (c) (right ordinate) Benzaldehyde formation with varying amounts of plasma without (□) and with (○) correction for the difference in standard peak areas.

there are no extraction steps in the assay, we have not used an internal standard. If desired, however, hexanal can be used as internal standard, the derivative of which elutes at about 10 min (Fig. 2). As hexanal is not stable under the present incubation conditions, it should be added after deproteinization but prior to centrifugation.

3.8. Reproducibility

Average values for plasma pools at the beginning and at the end of 18 consecutive assays were 321.1 ± 23.9 (C.V.% 7.4) and 334.5 ± 24.1 mU/l (C.V.% 7.2), respectively. The inter-assay variability was slightly less (5.5%).

3.9. SSAO activity in human plasma

In 51 healthy controls (age 49 ± 13 yr, 20 males) the measured SSAO activity was 352 ± 102 mU/l. Tufvesson [17] reported normal values of 471 ± 112 mU/l for serum amine oxidase activity in healthy adults, while Murphy et al. [28] reported average values of 305 ± 78 mU/l and 317 ± 84 mU/l for plasma amine oxidase in males and females, respectively. Other authors found average serum amine oxidase activities in adult controls of 18–32 McEwen units, which is equivalent to 167–296 mU/l [12–15]. We found no difference in SSAO activity between males and females.

From blood collected from 8 volunteers, serum and 5 different plasmas were prepared: heparinized-, heparinized with glutathione-, EDTA-, EDTA with trasylol-, and citrated-plasma. SSAO measurements in these various plasmas all gave similar results, varying between 2 and 5%; values determined in serum were however about 15% lower than in plasma.

The apparent K_m value of SSAO in plasma was determined in 9 different plasma samples and found to be 112 ± 18 μM, which compares well with previously reported values (110 μM [17], 110–120 μM [18], 225 μM [20]).

4. Conclusions

Described is a simple, sensitive and reproducible method for the measurement of SSAO activity in human plasma. A large number of samples can be processed in one day by one technician.

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