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Short Communication

Breakdown of 3,4-dihydroxybenzylamine and dopamine in plasma of various animal species by semicarbazide-sensitive amine oxidase

Frans Boomsma* and Gooitzen Alberts

Division of Internal Medicine I, Cardiovascular Research Institute COEUR, University Hospital Dijkzigt/Erasmus University, Dr. Molewaterplein 40, 3015 GD Rotterdam (Netherlands)

Mart M. Bevers

Department of Herd Health and Reproduction, Faculty of Veterinary Medicine, University of Utrecht, Utrecht (Netherlands)

Monique M. Koning

Division of Experimental Cardiology, Cardiovascular Research Institute COEUR, University Hospital Dijkzigt/Erasmus University, Dr. Molewaterplein 40, 3015 GD Rotterdam (Netherlands)

Arie J. Man in 't Veld and Maarten A. D. H. Schalekamp

Division of Internal Medicine I, Cardiovascular Research Institute COEUR, University Hospital Dijkzigt/Erasmus University, Dr. Molewaterplein 40, 3015 GD Rotterdam (Netherlands)

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ABSTRACT

We report a rapid breakdown of dopamine and especially of 3,4-dihydroxybenzylamine, the frequently-used internal standard in catecholamine determinations, in plasma of many but not all animal species. Species investigated were cow, sheep, goat, pig, horse, rabbit, dog, guinea pig, mouse, chicken, rat and man. In some species 3,4-dihydroxybenzylamine nearly completely disappeared at 4°C within 15 min after addition to the plasma. Added dopamine, but not norepinephrine and epinephrine, also rapidly disappeared at 4°C. Disappearance rates were increased at higher temperatures, and at 20°C also norepinephrine showed some breakdown. The breakdown is caused by a semicarbazide-sensitive amine oxidase in the plasma, and can be completely blocked by the addition of the inhibitor semicarbazide. Measurement of plasma catecholamine concentrations in animal species can thus lead to erroneous results, especially when 3,4-dihydroxybenzylamine is used as an internal standard. Only when blood is collected in tubes containing an inhibitor of semicarbazide-sensitive amine oxidase like semicarbazide can reliable plasma catecholamine measurements be performed.

* Corresponding author.

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INTRODUCTION

Plasma catecholamine concentrations are frequently determined by high-performance liquid chromatography with electrochemical detection (HPLC–ED). Prior to injection into the chromatographic system catecholamines are extracted from plasma by various means, *e.g.* with alumina, and 3,4-dihydroxybenzylamine (DHBA) is often used as an internal standard. Although the recovery of DHBA from human plasma is satisfactory, some authors have reported problems with the recovery of DHBA when using plasma of animal species such as horse and cow [1], dog [2,3], sheep [4] and goat [5]. The low recoveries found were attributed to protein binding or *in-situ* ion-pair formation. Methods proposed to solve the problem included varying the order of addition of reagents or simply switching to another internal standard.

Recently we found a rapid breakdown of dopamine (DA) in pig plasma, which could be attributed to the activity of a semicarbazide-sensitive amine oxidase (SSAO; EC 1.4.3.6) [6]. SSAO is one of the names given to a heterogeneous group of amine oxidases which have been reported to be present in various tissues and in plasma of various species (man, pig, rat, cow) [7–12]. Other names used include clorgyline-resistant amine oxidase, benzylamine oxidase or simply plasma oxidase [13–15]. Depending on the source of SSAO (tissue as well as species) properties such as substrate specificity may vary between species and tissue, but a common denominator is the resistance to inhibition by clorgyline and pargyline, and the sensitivity towards inhibition by carbonyl-group reagents like semicarbazide. The physiological substrate is not known, but benzylamine, tyramine and tryptamine are all good substrates.

Pig plasma SSAO oxidized DHBA even more readily than DA, but other catecholamines were not affected. The occurrence of similar SSAO activity in plasma of other animal species could be the explanation for the sometimes reported low DHBA recoveries and would lead to erroneous plasma catecholamine concentration measurements. We therefore investigated the stability of DHBA, of the natural catecholamines norepinephrine (NE), epinephrine (E) and DA, and of some synthetic catecholamines in plasma of various species (man, rat, chicken, mouse, guinea pig, dog, rabbit, horse, pig, goat, sheep and cow), both in the absence and presence of the SSAO inhibitor semicarbazide.

EXPERIMENTAL

Materials

The natural and synthetic catecholamine compounds were obtained from Sigma (St. Louis, MO, USA), except for epinine (a gift from the Zambon Group, Milan, Italy), α-methyldopamine (a gift from Merck Sharp & Dohme, Haarlem, The Netherlands) and dobutamine (a gift from Lilly Research Laboratories, Indianapolis, IN, USA). Semicarbazide was also obtained from Sigma.

Plasma

Blood was collected in chilled heparinized tubes containing 12 (10-ml tubes) or 5 (4-ml tubes) mg of glutathione and centrifuged immediately at 4°C (15 min, 3000 *g*). Plasma was stored at −70°C until assayed. Human blood was obtained from patients with essential hypertension. Animal blood was obtained from fawn-hooded rats, white Leghorn chickens, C57 black mice, Duncan–Hartley guinea pigs, Beagle dogs, New Zealand White rabbits, Dutch breeded horses, cross-bred Landrace × Yorkshire pigs, Saanen goats, Texel sheep and Holstein–Frisian cows.

Apparatus

The instrumentation for HPLC–ED consisted of a Spectra-Physics SP8800 low-presure gradient pump (San Jose, CA, USA) (used isocratically), a Kontron 460 autosampler (Milan, Italy) equipped with a 200-μl loop, an Antec CU-04AZ (Antec, Leiden, The Netherlands) electrochemical detector and a Merck-Hitachi D-2500 integrator (Tokyo, Japan). For HPLC with fluorimetric detection (HPLC–FD) a similar pump

and integrator were used, with a Marathon autosampler (Spark, Emmen, The Netherlands) equipped with a 50-μl loop and a Shimadzu RFR 535 spectrofluorimeter (Kyoto, Japan) (excitation at 350 nm, emission at 480 nm). Separations were performed on 3-μm Spherisorb ODS2 (100 mm × 4.6 mm I.D.) cartridges (Phase Separations, Deeside, UK) or 3-μm MicroSpher C_{18} (100 mm × 4.6 mm I.D.) columns (Chrompack, Bergen op Zoom, The Netherlands).

Assays

NE, E, DA, epinine, isoproterenol, α-methyldopamine, α-methylnorepinephrine and dobutamine were assayed by HPLC–FD after isolation from plasma by a specific liquid–liquid extraction method and derivatization with the selective fluorogenic agent 1,2-diphenylethylenediamine [16–18]. Epinine or α-methylnorepinephrine was used as an internal standard. NE, E, DA, DHBA and epinine were (also) assayed after isolation from plasma by liquid–liquid extraction and injection into the HPLC–ED system as described previously [16], using isoproterenol as internal standard. Usually the HPLC–ED and the HPLC–FD assays gave similar results for NE, E, DA and epinine. Occasionally the HPLC–ED assays appeared to give somewhat higher recoveries due to interfering peaks; in these cases the results of the HPLC–FD assays were used.

Experiments

Plasma samples (100–500 μl) were spiked with a mixture of catecholamines (1 ng each per ml of plasma) and incubated for various times at 4°C and at 20°C, both in the absence and presence of semicarbazide (final concentration 100 m*M*). After incubation the remaining concentrations of all catecholamines were measured and used to calculate the recoveries relative to the concentration at time zero. All experiments were performed in at least 3 different plasma samples of each species investigated.

RESULTS AND DISCUSSION

Fig. 1 shows the recoveries (mean ± S.D.) for DHBA, DA, NE and E in 12 different species after 4 h of incubation at 4°C and at 20°C. In the absence of semicarbazide, extensive loss of recovery was seen even at 4°C for DHBA in dog, rabbit, horse, pig, goat, sheep and cow plasma, which was even greater at 20°C. DA was less readily broken down, but still extensively at 4°C in pig, goat, sheep and cow plasma, and at 20°C also in guinea pig, dog, rabbit and horse plasma. At 4°C NE was stable in plasma of all animals tested, but at 20°C loss of recovery was also found for NE in pig, goat, sheep and cow plasma. At both temperatures tested E was fully recovered from all animal plasma samples. In the presence of 100 m*M* of semicarbazide the recoveries of all compounds were virtually complete after 4 h at 4°C and at 20°C, indicating that indeed the breakdown of the catecholamines was caused by an enzymatic process that could be inhibited by semicarbazide. Cow plasma showed recoveries of DHBA of 90 and 80% after 4 h at 4°C and at 20°C, respectively. Apparently the SSAO activity in cow plasma is so high that 100 m*M* of semicarbazide is not able to completely prevent the breakdown of DHBA. Other synthetic catecholamines tested (epinine, isoproterenol, dobutamine, α-methyldopamine and α-methylnorepinephrine) behaved like E and were similarly unaffected by SSAO.

The time-dependency of the oxidation of catecholamines by SSAO was investigated in plasma of 7 species of animals with SSAO activity and in human plasma, both at 4°C and at 20°C. Results are presented as mean recoveries of 2–3 experiments in Fig. 2. For the sake of clarity, standard deviations (all between 1 and 10%) are not included in the figures. The breakdown of DHBA was strikingly fast in plasma of cow, sheep, goat and pig: even at 4°C nearly all DHBA had been converted within 15 min. In plasma of horse, rabbit and dog the SSAO activity was less, but still led to extensive breakdown of DHBA even at 4°C. At 20°C the conversions proceeded faster. A similar pattern was seen with DA, albeit at a slower rate, indicating again that DA is a less good substrate than DHBA. NE remained intact at 4°C, but at 20°C a decrease in recovery is clear-

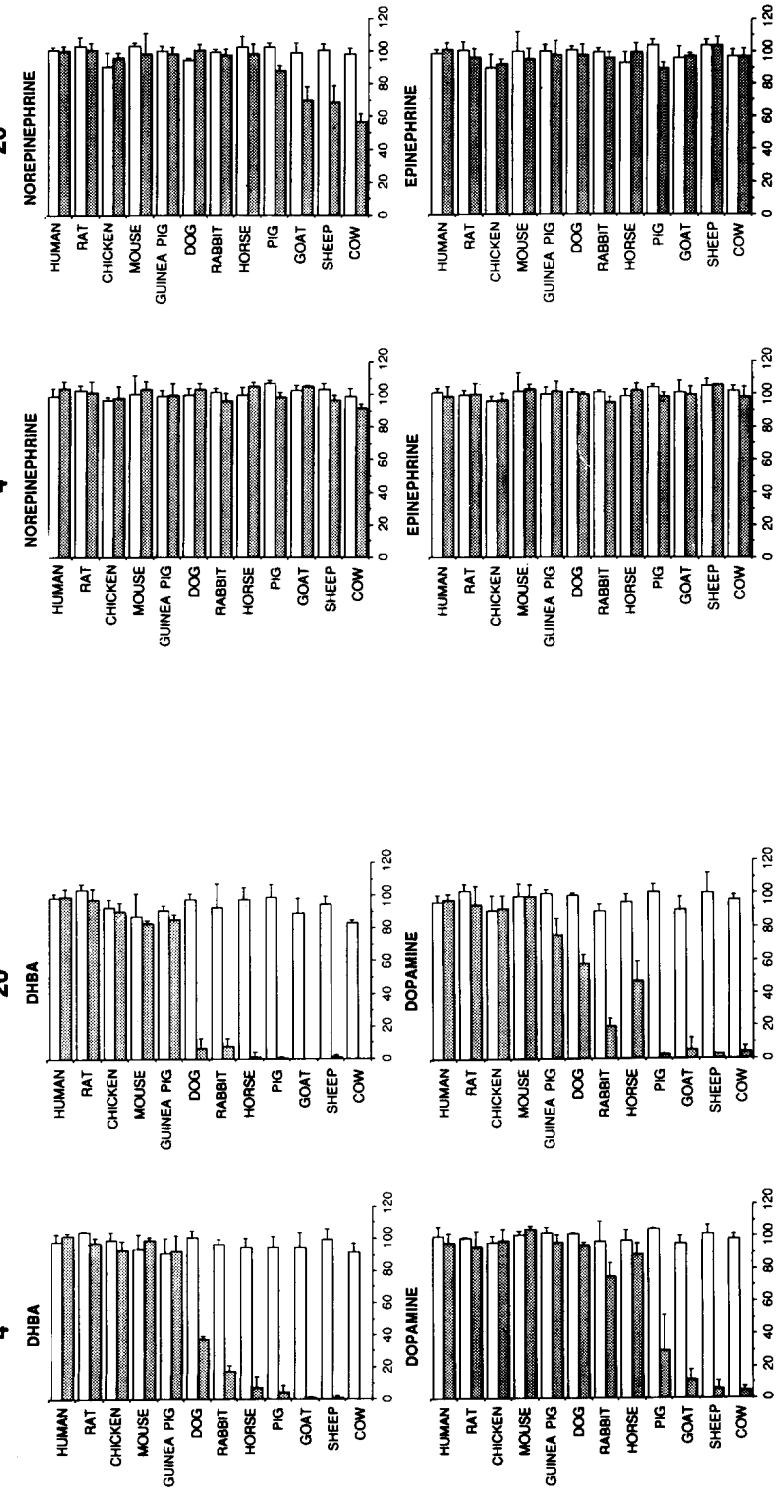

Fig. 1. Recoveries of 3,4-dihydroxybenzylamine (DHBA), dopamine, norepinephrine and epinephrine, added to plasma of various animals, after 4 h of incubation at 4°C and at 20°C in the absence (hatched bars) or in the presence (blank bars) of semicarbazide (100 m*M*). Recoveries are expressed as a percentage of the amounts at time zero and are mean ± standard deviation of incubations with at least 3 plasma samples of each species.

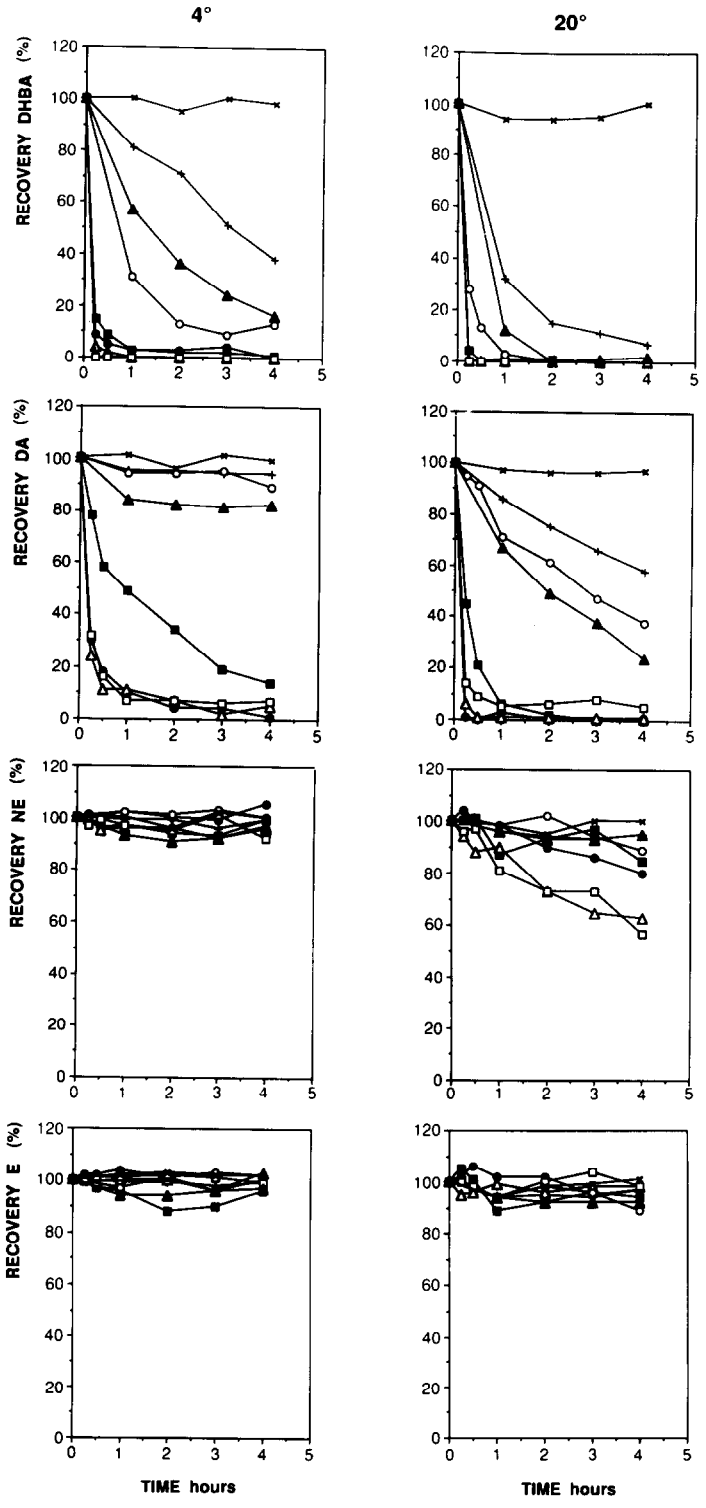

Fig. 2. Recoveries of 3,4-dihydroxybenzylamine (DHBA), dopamine (DA), norepinephrine (NE) and epinephrine (E) after various times of incubation at 4°C and at 20°C in plasma of various species. Cow (□), sheep (△), goat (●), pig (■), horse (○), rabbit (▲), dog (+), man (×). Recoveries are expressed as a percentage of the amounts at time zero.

ly seen in plasma of animals with the highest rate of breakdown of DHBA and DA. E remained unaffected by SSAO at both temperatures.

Our results clearly indicate that plasma of many species contains SSAO activity. Only in human, rat, mouse and chicken plasma could no SSAO activity be detected. This contrasts with previous reports on the presence of SSAO activity in human plasma and in rat tissues [9,12,13]. The differences in SSAO activity between the plasmas of different animals might of course be due to a variation in the concentration of the enzyme, but in view of the reported heterogeneity of the SSAO from different origins it might as well be a result of differences in the SSAO enzymes involved. The pH of all our plasma samples varied from 7.6 to 8.0, which may have had a minor influence on SSAO activity (optimal pH for SSAO is reported to be between 7 and 9, depending on species and substrate). Indications for heterogeneity of SSAO are supported by our findings that, (1) in contrast to the general conclusion that DHBA seems to be a better substrate for the enzyme than DA, in guinea pig plasma some loss of DA was observed at 20°C, while no significant loss of DHBA was found, and (2) DHBA is oxidized faster in horse plasma than in rabbit plasma, while the reverse is true for DA.

The plasma enzyme is remarkably active towards DHBA and DA even at 4°C. Obviously, this leads to serious problems in plasma catecholamine measurements. Our results agree with the few reports in the literature where the loss of recovery of DHBA was mentioned, although the underlying mechanism turns out to be quite different than suggested in these reports, *i.e.* SSAO activity instead of ion-pair formation [1] or protein binding [2,5]. The unsuitability of DHBA as an internal standard can be overcome by switching to another compound [1,4,5] which is not or less affected by SSAO, but then the problem of the breakdown of DA (as noticed by Hardee *et al.* [1] and Garty *et al.* [5] in cow and goat plasma) still remains. Varying the order of adding reagents as advocated before [2,3] may reduce the time of contact between SSAO and its substrates, but is no real solution. It thus seems necessary to inhibit the SSAO activity with an inhibitor like semicarbazide for reliable measurements of catecholamines in animal plasma. Deproteinization of plasma as suggested by Garty *et al.* [5] to prevent supposed protein binding of DHBA would of course also prevent SSAO to be active. However, because of the inevitable time-lag between blood collection and deproteinization, and of the extra work, it seems preferable to collect blood from animals in tubes containing semicarbazide to prevent the problems associated with SSAO activity.

CONCLUSIONS

Plasma of many animal species (cow, sheep, goat, pig, horse, rabbit, dog, guinea pig) contains SSAO activity, which can rapidly break down DHBA and DA even at 4°C. For reliable measurements of plasma catecholamines in these species it is necessary to collect blood in tubes containing an SSAO inhibitor like semicarbazide.

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