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Photostability of epinephrine – the influence of bisulfite and degradation products

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Received August 18, 2003, accepted October 10, 2003

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Pharmazie 59: 457–463 (2004)

Sulfites are previously demonstrated to increase the photodegradation of epinephrine. The aim of this study was to clarify the factors responsible for this effect. Adrenochrome sulfonate seems to be the important substance. Photoproduction of singlet oxygen is indicated to be the mechanism by which adrenochrome sulfonate acts. Protection of epinephrine solutions from irradiation < 418 nm prevented the photodegradation. A reaction pathway for the photochemical decomposition of epinephrine in the presence of bisulfite is suggested.

1. Introduction

Epinephrine is frequently added to injections and infusions. It induces vasoconstriction that helps to keep other drugs localised at the site of injection. This effect is frequently utilised in anaesthetic preparations and in epidural analgesic solutions (Niemi and Breivik 1998). Transparent infusion bags containing epinephrine can be exposed to both indirect sunlight and room light for several days at the hospital ward (Tønnesen and Karlsen 1995). Exposure to irradiation can further occur during manufacturing, packing and storage of the products.

Solutions of epinephrine are frequently prepared in combination with sulfites to prevent oxidation of the catecholamine. However, previous studies have shown that sodium metabisulfite can markedly decrease the stability of epinephrine when exposed to glass filtered sunlight (Brustugun et al. 2000). The present work was undertaken to obtain further information on the parameters of importance for the photodestabilizing effect of bisulfite on epinephrine. The effect of degradation products from epinephrine on the degradation process has been of particular interest.

2. Investigations and results

2.1. The effect of bisulfite and adrenochrome on the degradation process

A previous investigation has shown that epinephrine in an epidural analgesic solution containing bisulfite is photolabile compared to solutions without bisulfite (Brustugun et al. 2000). This observation was made although none of the ingredients in the preparation absorbed irradiation in the actual exposure range (>310 nm). This indicated that some other substance (e.g. degradation products or impurities) is involved in the process. The thermal degradation of epinephrine is a complex process including many intermediates of varying stability (Heacock 1959; Heacock and Powell 1973; Bors et al. 1975; Gohn et al. 1976a; 1976b; Bors et al. 1978; d'Ischia et al. 1988). One major thermal degradation product is adrenochrome. This compound is quite stable compared to many of the other degradation products of epinephrine. Besides thermal degradation light can also induce degradation of epinephrine and similar substances (Hoevenaars 1965; Gohn et al. 1976a; 1976b; Jahnke and Frenkel 1978; Wollmann and Grunert 1984; d'Ischia and Prota 1987; Polewski and Slawinska 1990; Albini and Fasani 1998). Adrenochrome is further reported to be a photodegradation product of epinephrine formed both in direct and sensitized reactions (de Mol et al. 1979a; 1979b; Kruk 1985). This is of interest because adrenochrome absorbs light with a λ_{max} at 480 nm. Light of wavelengths about 480 nm can penetrate window glass.

In order to study the effect of bisulfite and the degradation product adrenochrome on the photostability of epi-

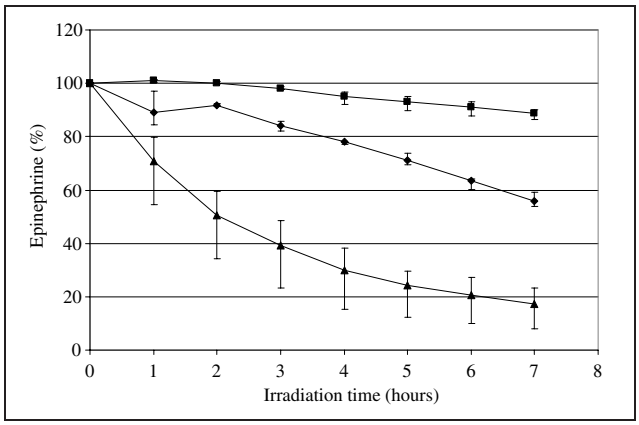


Fig. 1: Remaining percent epinephrine, 2.0 μg/ml, in the presence of various additives during exposure in the Suntest CPS ($\lambda > 310$ nm) for 7 h. Solutions containing sodium metabisulfite, 1.8 μg/ml (—◆—), a combination of sodium metabisulfite, 1.8 μg/ml and adrenochrome, 1,0 μg/ml (—▲—) or only adrenochrome, 1.0 μg/ml (—■—). (n = 3, average ± max/min)

nephrine three types of test solutions were made as described in section 4.3.2. Solutions containing epinephrine were prepared in the presence of bisulfite, adrenochrome or a combination of bisulfite and adrenochrome, respectively. The solutions were exposed to a continuous irradiation source (310–800 nm) for 7 h. As can be seen (Fig. 1) the solutions containing only adrenochrome as an additive were quite stable throughout the period of irradiation and 90% of the epinephrine remained after the 7 h of exposure. This is consistent with previous results (Brustugun et al. 2000). This indicates that adrenochrome alone has little, if any, effect on the photoinduced degradation of epinephrine. However, the epinephrine solutions containing both bisulfite and adrenochrome showed a rapid degradation of epinephrine. At the end of the experiment only ~10% of the drug remained. The epinephrine solutions containing only bisulfite did also show degradation of epinephrine, but at a lower rate than the solutions initially containing the combination with adrenochrome. It therefore seems likely that bisulfite and adrenochrome have a synergistic effect on the degradation of epinephrine.

2.2. The reaction of adrenochrome with bisulfite

The results indicate a synergistic effect between bisulfite and adrenochrome on the photodegradation of epinephrine. Adrenochrome could be suspected to react with bisulfite to form a new substance. The formation of such a new substance is indeed reported earlier: i.e. the formation of adrenochrome sulfonate (van Espen 1958; Mattok et al. 1966; Marchelli et al. 1971; Heacock and Powell 1973). The UV-absorption maxima that have been reported for the adrenochrome sulfonate in aqueous solution are: 360 nm and 350 nm (van Espen 1958; Mattok et al. 1966). To investigate the reactivity of a mixture of epinephrine, adrenochrome and bisulfite in solution, samples were made as described in section 4.3.3. UV-Vis-spectra of the samples containing only epinephrine (100 μg/ml) and adrenochrome (12 μg/ml, i.e. 67 μM) were recorded. Sodium metabisulfite (3.7 μg/ml, i.e. 38.9 μM as bisulfite) was then added and new spectra were recorded at 15, 45, 90, 120 min and again after 20 h (Fig. 2). The solutions were stored in the dark during the experiment.

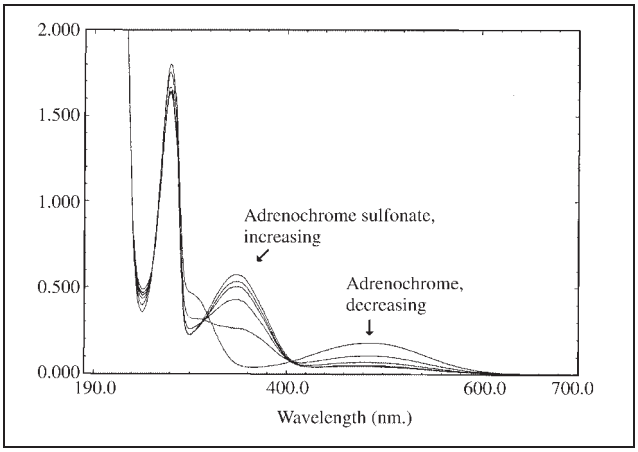


Fig. 2: The absorption spectra of a solution containing epinephrine (100 μg/ml) and adrenochrome (12 μg/ml) before and after addition of sodium metabisulfite (3.7 μg/ml). The spectra are recorded before the addition of sodium metabisulfite and after 15, 45, 90, 120 min and then again 20 h after the addition of bisulfite. The abs. max. at 350 nm is increasing with time and the abs. max. at 480 nm is decreasing with time.

After the addition of bisulfite a new absorption maximum appears at 350 nm (Fig. 2). This is likely due to the formation of adrenochrome sulfonate. Degradation products of adrenochrome absorbing in the same spectral range could also be considered. Such substances are reported in the literature. One is 5,6,-dihydroxy-*N*-methyl indole (λ_{max} 340 nm) which is a degradation product of adrenochrome formed by hydrogen peroxide in alkaline solution (i.e. above pH 8,5) (Bors et al. 1978). Two others are 5,6-dihydroxy-1-2,3-indoledion (λ_{max} 363 nm and 509 nm) and a dimer of the latter (λ_{max} 352 nm and 512nm) (d'Ischia et al. 1988), both formed from adrenolutin (λ_{max} 317 nm and 393 nm) – a known degradation product of adrenochrome.

Formation of a distinct absorption maximum above 500 nm could, however, not be observed in our experiments. It is also unlikely that the above secondary degradation products are formed to a large extent under the present experimental conditions. The reaction between bisulfite and adrenochrome is reported to take place very rapidly and adrenochrome sulfonate is expected to be formed almost instantly (Mattok et al. 1966). The fact that the new absorption maximum is recorded shortly after the addition of bisulfite therefore points in the direction of adrenochrome sulfonate formation. The absorption maximum at 350 nm is consistent with the absorption spectrum of adrenochrome sulfonate. From these considerations it seems likely that adrenochrome sulfonate is the main sensitizer formed *in situ* in the epinephrine preparations.

2.3. Effect of wavelength on the degradation of epinephrine

The difference in the absorption spectra of adrenochrome and adrenochrome sulfonate was used to obtain further information on the influence of adrenochrome sulfonate in the photodegradation of epinephrine. Adrenochrome has absorption maxima (λ_{max}) at 480 nm and at 310 nm, and an absorption minimum (λ_{min}) at 360 nm. Adrenochrome sulfonate on the other hand does not absorb light at 480 nm but has an absorption maximum (λ_{max}) at 350 nm. Samples were prepared containing epinephrine (2.0 μg/ml) and adrenochrome (1.0 μg/ml) as outlined in section 4.3.4. Three of the four solutions also contained sodium

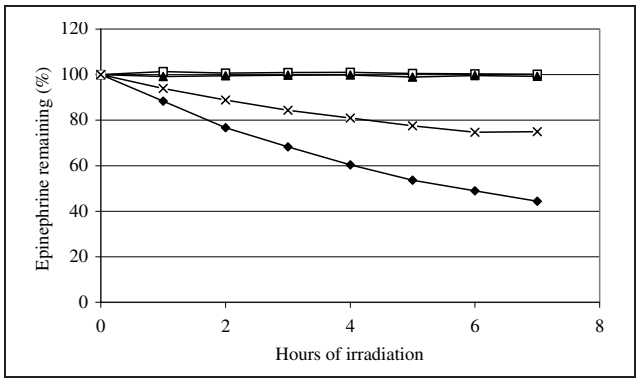


Fig. 3: The effect of wavelength (350 nm vs. 480 nm) and oxygen content on the degradation of epinephrine (2.0 μg/ml) in solution with adrenochrome (1.0 μg/ml) in the absence and presence of sodium metabisulfite (1.8 μg/ml). The curves represent: solution containing bisulfite irradiated at 350 nm (—◆—), solution not containing bisulfite irradiated at 350 nm (—□—), solution containing bisulfite irradiated at 480 nm (—▲—) and solution containing bisulfite irradiated at 350 nm and purged with nitrogen (—×—).

metabisulfite (1.8 μg/ml). The solutions were exposed to a monochromatic irradiation source (350 ± 10 nm or 480 ± 10 nm). From Fig. 3 it appears that the solution without bisulfite is stable under the given conditions when irradiated at 350 nm. The solution containing bisulfite irradiated at 480 nm was also stable. However, the solution containing bisulfite irradiated at 350 nm was degraded markedly during the seven hours of exposure. This again emphasizes that adrenochrome sulfonate is likely to be the sensitizer responsible for the photodegradation of epinephrine.

Oxygen radicals often play an important role in photodegradation reactions. A sample containing bisulfite was irradiated at 350 nm under constant nitrogen flushing to lower the oxygen concentration in the medium. This had a retarding effect on the degradation process. The result indicates that oxygen takes part in the photodegradation of epinephrine. Some epinephrine is, however, still decomposed, despite the purging with nitrogen. This may be due to trace amounts of oxygen still remaining in the sample or other reactions not involving oxygen.

2.4. The effect of radical scavengers and quenchers on the photodegradation of epinephrine

The effect of nitrogen purging on the photostability of epinephrine indicated some involvement of oxygen in the degradation process. To obtain further information on the reaction mechanism solutions of epinephrine (2.0 μg/ml) were prepared as described in section 4.3.5. Various radical scavengers and quenchers were added and the solutions were irradiated by monochromatic light or exposed to a continuous irradiation source. The following scavengers and quenchers were selected: t-butanol and d-mannitol as scavengers of the hydroxyl radical ($OH^{\bullet}$), superoxide dismutase (SOD) as scavenger for the superoxide anion radical ($O_2^{\bullet-}$), and 2,5-dimethyl furan, 1,4-Diazadicyclo[2.2.2]-octane and sodium azide as quenchers of singlet oxygen ($^1O_2$). For further detection of singlet oxygen the chemiluminescence probe trans-methoxyvinylpyrene (t-MVP) was applied to solutions of adrenochrome sulfonate.

No inhibitory effect was observed by addition of t-butanol, d-mannitol or SOD to samples exposed to a continuos irradiation source (Suntest CPS). On average 70.6% epinephrine (68.8–72.8%, $n = 12$) remained after irradiation independent of the content of scavengers. A slightly stabilizing effect was observed after addition of 2,5-dimethyl furane. The amount of epinephrine remaining after exposure was 78.5% (77.0–81.1%) indicating some effect of the singlet oxygen quencher.

None of the scavengers or quenchers seemed to have a clear inhibitory effect on the epinephrine degradation when the samples were irradiated at 350 ± 10 nm. The quenchers sodium azide and DABCO rather seemed to have a destabilising effect on epinephrine. The solutions containing DABCO showed a decrease in the epinephrine concentration of 30% even before the irradiation was initiated. Azide demonstrated a catalytic effect on the photodegradation of epinephrine. DABCO has previously been used, apparently without problems, by others in the study of epinephrine (Kruk 1985). The reason for this inconsistency in results is not known. When using the chemiluminescence probe trans-methoxyvinylpyrene (t-MVP) the absorption spectrum indicated a ground state interaction between t-MVP and adrenochrome sulfonate. This probe was therefore not considered suitable in these experiments.

2.5. The detection of singlet oxygen by luminescence

The effect of 2,5-dimethyl furane indicated that singlet oxygen ($^1O_2$) may take part in the photodegradation process. To investigate this further four solutions containing various amounts of adrenochrome sulfonate (in the concentration range 13.0 μM to 22.0 μM) were prepared in deuterated water (as described in section 4.3.6). The absorbance of the samples at 350 nm was measured to confirm the formation of adrenochrome sulfonate. The absorbance of the samples ($n = 4$) at 350 nm was in the range 0.16–0.30 AU.

The samples were excited at 350 nm and the presence of singlet oxygen was measured by direct detection of luminescence at 1270 nm in a steady state mode. A plot of the signal detected at 1270 nm (area under the curve) versus the absorbance of the sample at 350 nm showed good correlation ($r^2 > 0.99$, data not shown). This indicates that singlet oxygen is generated by adrenochrome sulfonate during irradiation at 350 nm. A signal could also be detected at 1270 nm when solutions were prepared in distilled water and 5 mM phosphate buffer. When epinephrine was added to the samples in distilled water this signal was quenched to some extent. The quenching was a function of epinephrine concentration. This indicated that epinephrine interacted with the singlet oxygen generated by adrenochrome sulfonate.

2.6. The effect of the concentration of adrenochrome sulfonate on the photodegradation of epinephrine

Various amounts of sodium metabisulfite (19.6 μM, 9.8 μM and 4.9 μM) were added to samples containing 100.0 μg/ml (0.6 mM) epinephrine and 12 μg/ml (67.0 μM) adrenochrome. Two molecules of bisulfite are formed from one molecule of sodium metabisulfite. At pH 4.7 the dimerisation constant (K_D) for the equilibrium ($2HSO_3^- \leftrightarrows S_2O_5^{2-}$) is $0.07\ M^{-1}$ (Connors 1986). Most of the metabisulfite should therefore be present as bisulfite in the test solutions. As a result of the equilibrium bisulfite is constantly formed from metabisulfite when bisulfite is consumed in the reaction with adrenochrome. Under the given experimental conditions adrenochrome is in molar excess of bisulfite. The formation of adrenochrome sulfonate is thereby restricted by the total amount of bisulfite. The absorbance at 350 nm was used as a measure of the formation of adrenochrome sulfonate. The concentrations of adrenochrome and bisulfite were selected to give an amount of adrenochrome sulfonate corresponding to an absorbance of < 0.7 AU at 350 nm to avoid inner filter effect.

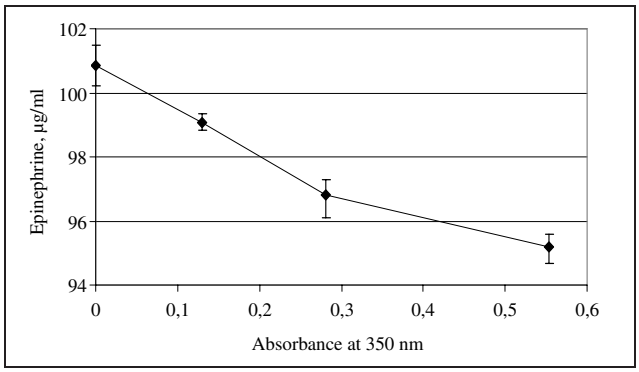


Fig. 4: Reduction in concentration of epinephrine (μg/ml) as a function of the concentration of adrenochrome sulfonate (determined by absorbance at 350 nm) (average ± max/min, $n = 3$) during irradiation (350 ± 10 nm) for 75 min. The absorbance contribution of adrenochrome at 350 nm was corrected for.

Table 1: Consumed oxygen and epinephrine after 75 min exposure (350 ± 10 nm)

Initial concentration of epinephrine (μg/ml)	Remaining epinephrine (μg/ml)	Consumed O_2, (μg/ml)	Molar ratio consumed O_2 to photodegraded epinephrine
200.5 (200.7–204.1)	190.8 (190.1–191.5)	1.5 (1.4–1.6)	1.09
100.8 (100.2–101.6)	95.2 (94.7–95.6)	1.3 (1.2–1.3)	1.32
50.9 (50.5–51.1)	47.2 (46.8–47.6)	1.1 (1.0–1.3)	1.69
25.2 (25.1–25.3)	23.0 (23.0–23.1)	0.7 (0.7–0.7)	1.82

(n = 3, average ± max/min values)

The degradation of epinephrine seemed to be a function of the concentration of adrenochrome sulfonate when the samples were irradiated at 350 nm (Fig. 4). The pH did not change substantially during the experiment (4.67–4.73). The consumption of oxygen was monitored throughout the experiment by an oxygen sensitive electrode. Correlation between consumption of oxygen and irradiation time was observed ($r^2 \geq 0.94$). The molar ratios of consumed oxygen (O_2) to degraded epinephrine were between 1.22 and 1.54 on solutions containing 100.0 μg/ml.

The UV absorption data at 350 nm were further used to estimate changes in concentration of adrenochrome sulfonate as a function of irradiation. Only a minor decrease (3.2–6.6%) in absorption was observed and the number of moles epinephrine decomposed were not correlated to this decrease. This indicates that adrenochrome sulfonate was not consumed during the experiment.

As can be seen in Table 1 the molar ratio of consumed O_2 to photodegraded epinephrine is 1.82 when the initial concentration of epinephrine is 25 μg/ml. This is in agreement with the previous results reported by Jahnke and Frenkel for the methylene blue sensitized photodegradation of epinephrine (Jahnke and Frenkel 1978). As the initial concentration of epinephrine is increased, the ratio was decreased, indicating a concentration dependent shift in the reaction pathway.

2.7. The effect of UV-vis filter on the photostability of epinephrine

Sulfites are antioxidants that are very widely used in preparations of epinephrine. Although it is desired to avoid sulfites in parenteral preparations, both for stability reasons and for medical reasons, it is not always easy to find a suitable replacement. Preparations can possibly be made without sulfites, but then a reduction in shelf-life will have to be considered. Purging of solutions with nitrogen is another possibility. This is suitable for injections, but not for larger volumes like infusion bags. Yet another approach would be to use a suitable physical protection of the infusion solutions (i.e. a coloured infusion bag or an outer protection). The device must however be transparent in order to allow for visual inspection of the preparation.

In order to determine the effect of such a device samples were prepared as described in section 4.3.8, containing 2 μg/ml epinephrine, and irradiated according to the requirements of the ICH Guidelines (ICH 1995). Some of the solutions were protected by a filter (cut-off 418 ± 10 nm) during the exposure. The results presented in Table 2 show that a yellow filter has a retarding effect on the photodegradation reaction.

Table 2: The effect of a protection filter (cut-off 418 ± 10 nm) on the photodegradation of epinephrine (Average ± max/min values)

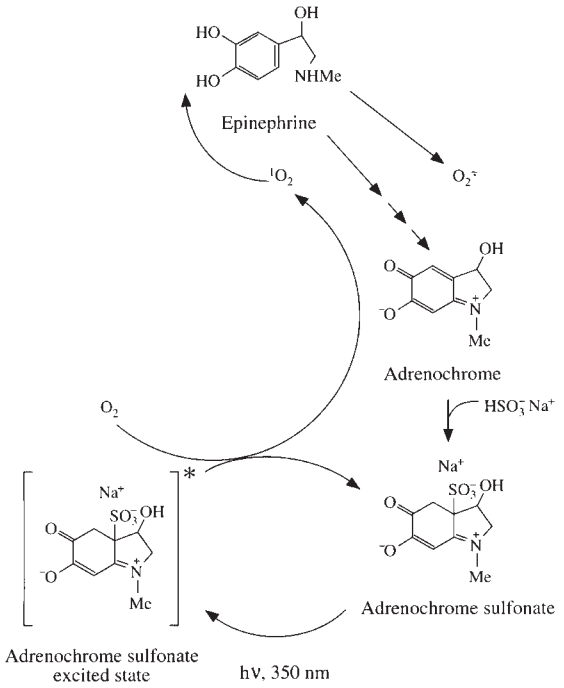
Sodium metabisulfite (1.8 μg/ml), present (+) or absent (−)	Initial concentration (μg/ml) of epinephrine (n = 4)	Remaining epinephrine (%), 2.9 h exposure, (n = 2)	Remaining epinephrine (%), 7.1 hours exposure, (n = 2)
With filter			
(−)	1.99 (1.99–1.99)	96.5 (96.5–96.6)	94.7 (94.3–95.1)
(+)	1.97 (1.97–1.97)	96.6 (96.5–96.6)	92.3 (91.9–92.7)
Without filter			
(−)	1.98 (1.98–1.99)	92.8 (92.7–92.9)	84.5 (84.4–84.6)
(+)	1.97 (1.97–1.97)	81.7 (81.5–81.9)	58.9 (58.6–59.2)

3. Discussion

A previous study has demonstrated that bisulfite contributes to the photodegradation of epinephrine in solution (Brustugun et al. 2000). This effect is confirmed through the experiments in the present work. The degradation process is observed to take place by exposure at 350 ± 10 nm, a wavelength at which adrenochrome sulfonate is the only absorbing component in the sample. Thus, the catalytic effect of bisulfite on epinephrine photodecomposition seems to be due to the formation of adrenochrome sulfonate which acts as a sensitizer in the photodegradation process.

The degradation induced by adrenochrome sulfonate may partly be ascribed to the generation of singlet oxygen. The luminescense signal detected at 1270 nm, characteristic of singlet oxygen, shows a good correlation with the concentration of adrenochrome sulfonate in solutions

Scheme 1



Postulated photodegradation pathway of epinephrine in the presence of bisulfite.

(2.5). This indicates that adrenochrome sulfonate is a source of reactive singlet oxygen molecules. Involvement of singlet oxygen in the sensitized photodegradation of epinephrine has previously been demonstrated for other sensitizers: methylene blue (Jahnke and Frenkel 1978), rose bengal and fluoresceine (Kruk 1985). Kruk postulated that singlet oxygen leads to the oxidation of epinephrine to adrenochrome. It is possible that adrenochrome sulfonate causes epinephrine degradation by the same mechanism.

The influence of singlet oxygen was also emphasized by the quenching effect of 2,5-dimethyl furane on the photodegradation of epinephrine when exposed to a continuos irradiation source (2.4). Based on these results a degradation pathway could be postulated (Scheme 1).

The lack of effect of SOD on the photodegradation of epinephrine indicates that superoxide is not important in the degradation process although it should be noted that the pH in the experiment (pH 4.7) was below the optimum range for SOD (pH 5.5–9.5). Previously Jahnke and Frenekl used SOD in comparable concentrations but at higher pH (pH 7.8) and observed an inhibitory effect on the photodegradation of epinephrine (Jahnke and Frenkel 1978). Superoxide has been linked to autooxidation of epinephrine at pH > 8 (Bors et al. 1978) but this process may not be favoured at pH 4,7 as the rate constant for the dismutation of superoxide has its maximum at pH 4,8 (in the order of 10^7 $M^{-1}s^{-1}$) (Bielski and Cabelli 1995). In a system containing sulfites superoxide could also react with the sulphite and produce highly reactive hydroxyl radicals (Stewart and Tucker 1985). It has previously been stated that this theoretically could be of importance in the sulfite induced photodegradation of epinephrine (Brustugun et al. 2000). In the present experiments the hydroxyl radical scavengers, d-mannitol and t-butanol, did not seem to have any effect on the degradation process. The possible formation of hydroxyl radicals therefore seems to be of minor, if any importance to the process.

Kruk has postulated that the sensitised photodegradation of epinephrine mainly follows an oxygen independent pathway (Kruk 1985). The effect of nitrogen purging on the present results (Fig. 3) indicates that an oxygen independent pathway may be favoured under certain experimental conditions.

A correlation was observed between the amount of epinephrine decomposed and the amount of oxygen consumed (Table 1). The degradation product adrenochrome contains no more oxygen atoms than the parent compound, epinephrine. Thus, singlet oxygen seems to interact with epinephrine (EPI) to form adrenochrome without being consumed in this reaction. Oxygen is, however, consumed in the degradation process; possibly through formation of superoxide (Scheme 2), leading to the formation of hydrogen peroxide and eventually water. This hypothesis does, however, need to be confirmed by further experiments. Cytochrome c could be a candidate for use as a superoxide indicator, but is not suitable because it is not only reduced by superoxide, but also by intermediates in the epinephrine degradation reaction (Fridovich 1985).

Scheme 2

$$EPI + {}^1O_2 \rightarrow EPI\cdot + HO\cdot_2 \quad \text{eq. (1)}$$

$$H^+ + O\cdot_2^- \leftrightarrows HO\cdot_2 \quad \text{eq. (2)}$$

$$EPI\cdot + O_2 \rightarrow EPIQ + HO\cdot_2 \quad \text{eq. (3)}$$

$$EPI + O\cdot_2^- \rightarrow EPI\cdot + H_2O_2 \quad \text{eq. (4)}$$

$$EPI\cdot + O\cdot_2^- \rightarrow EPIQ + H_2O_2 \quad \text{eq. (5)}$$

Possible reactions in the oxygen dependent degradation of epinephrine. Eqs. (3)–(5) were postulated by Bors et al. (1978) (EPI· is the epinephrine semiquinone, and EPIQ is the epinephrine quinone)

The possible influence of the postulated reaction (Eq 1) in the sensitized photodegradation of epinephrine has also been discussed by Kruk (1985). The species EPI was first postulated by Bors et al. (1978), and the reaction of epinephrine (EPI) with singlet oxygen ($^1O_2$) was also postulated by Jahnke and Frenkel (1978). The results presented in Table 1 show a decrease in the ratio of consumed O_2 to decomposed epinephrine as the initial concentration of epinephrine was increased. A concentration dependent shift in the reaction pathway could in theory account for this. The concentration of adrenochrome sulfonate is the same in all solutions, and since the compound apparently not is photodegraded its concentration is virtually constant throughout the irradiation time. Thus, the production of singlet oxygen ($^1O_2$) should be the same in all solutions. At low concentrations of epinephrine the process is thought to proceed by Eq 1 (consuming one molecule of oxygen) and Eq 3 (consuming another molecule of oxygen) (Jahnke and Frenkel 1978). As the initial concentration of epinephrine increases, however, reactions 4 and 5 may become more important. Epinephrine and epinephrine semiquinones (EPI·) can, with increasing concentration, more frequently interact with the short-lived superoxide radical (O^-_2) from Eq 1 and 2. The process involves just one molecule of oxygen and this can lower the ratio oxygen consumed vs. epinephrine degraded. Superoxide dismutase did not seem to have an inhibitory effect on the photodegradation of epinephrine (section 2.4). The reason for this is thought to be the low initial concentration of epinephrine (2.0 μg/ml) favouring the process by eqs. (1) and (3).

In addition to the oxygen dependent degradation pathway (Scheme 2) a non-oxygen dependent pathway has been indicated. This could possibly be caused by a direct effect of exited adrenochrome sulfonate on epinephrine leading to the decomposition of the latter. This hypothesis does, however, need to be confirmed by further experiments.

For practical purposes it should be noted that epinephrine is unstable in solution and therefore trace amounts of adrenochrome will be formed through the shelf life of a pharmaceutical preparation. Sulfites are widely used as stabilisers in these preparations and will initially be in excess of adrenochrome. As the reaction between adrenochrome and bisulfite is a fast reaction (Mattok et al. 1966), the formation of adrenochrome sulfonate in pharmaceutical products under storage and use seems likely. To prevent its action as photosensitizer a suitable outer container or coloured infusion bag could be used. Experiments showed that a filter with cut-off at 418 ± 10 nm had a protective effect on the samples. The main concern when selecting the appropriate container would be to exclude the irradiation absorbed by adrenochrome sulfonate (λ_{max} 350 nm) while maintaining the transparency of the outer container. As the formation of adrenochrome and thereby adrenochrome sulfonate in the presence of bisulfite can not be avoided in common preparations of epinephrine, a protective container with a cut-off > 400 nm should be used on a routine basis during storage and administration.

4. Experimental

4.1. Materials

Adrenochrome, epinephrine bitartrate (reference standard, Sigma), d-mannitol, sodium azide, 1-octanesulfonic acid, superoxide dismutase (SOD, from bovine erythrocytes) were all purchased from Sigma Chemical, St. Louis, USA. 1,4-Diazadicyclo[2.2.2]-octan 97% (DABCO) was purchased from Aldrich-Chemie, Steinheim, Germany. Potassium dihydrogen phosphate (p.a.) and sodium chloride (p.a.) were both purchased from Merck, Darmstadt, Germany. Potassium hydroxide was supplied by Eka Nobel, Bohus, Sweden. Epinephrine bitartrate, sodium edetate, sodium chloride and sodium metabisulfite were all supplied by Norsk Medisinal Depot, Oslo, Norway. Tert-butylalcohol (puriss p.a.) was supplied by Fluka AG, Switzerland. Deuterium oxide (D_2O) was supplied by Euriso-top, Gif-sur-Yvette, France. Trans-methoxyvinylpyrene (t-MVP) was supplied by Molecular Probes Inc., Eugene OR, USA.

4.2. Instrumentation

4.2.1. HPLC analysis

The concentration of epinephrine was measured by reversed phase HPLC. The analysis was performed using a 100 mm × 4,6 mm, 5 μm diphenyl column (LC-DP, Supelco, Bellefonte PA, USA). The mobile phase was composed of 1-octanesulfonic acid (5 mM), methanol (6%), acetonitrile (2%) and acetic acid (0.05%) in 5 mM KH_2PO_4. The flow rate was set to 1.0 ml/min using a Shimadzu LC-9A pump. Epinephrine was detected at 280 nm (Shimadzu UV-Vis Detektor SPD-10A). The samples were injected using a Shimadzu autoinjector SIL-9A. Data acquisition was completed using a Shimadzu C-R3A integrator. The retention time for epinephrine was approximately 5.5 min. Validation of the method gave a linearity of $r^2 > 0.999$ in the concentration range 0.1 μg/ml–10 μg/ml. The system precision was found to be ± 0.15% (2 μg/ml epinephrine, n = 6). The mean intra day coefficient of variation was found to be 0.25% (2 μg/ml epinephrine, n = 6). The mean inter-day coefficient of variation between two days was determined to be 0.20% (2 μg/ml epinephrine, n = 6). The limit of detection was estimated to be 2.5 ng/ml. The limit of quantitation was estimated to be 25 ng/ml.

4.2.2. UV-Visible spectrophotometer

For UV-Vis absorbance measurements a Shimadzu UV-260 UV-Visible recording spectrophotometer was used.

4.2.3. Luminescence detection

The generation of singlet oxygen ($^1O_2$) was measured by direct detection of singlet oxygen luminescence at 1270 nm in a steady state mode made by means of a fluorescence system using Felix™ for Windows softweare. The exitation source was a 75 W xenon lamp. The monochromators were Model 101 (f/4 0.2 – meter Czerny-Turner configuration), The detector was a EQ – 817 Germanian Detection System operated under liquid nitrogen conditions.

4.2.4. Irradiation conditions (continuous irradiation source)

Photochemical stability testing by means of a continuous irradiation source (310 nm–800 nm) was performed in a Suntest CPS, Heraeus GmbH, Hanau, Germany. The Suntest was equipped with a 1.8 kW xenon lamp with a maximum effect of 765 W/m^2. The samples in the Suntest were exposed in polypropylene test tubes (KEBO Lab, Oslo, Norway, cat.no.) unless other is specified.

4.2.5. Irradiation conditions (monochromatic irradiation source)

Exposure to monochromatic irradiation was obtained by use of a Monochromator f 3.4, 900 W xenon arc lamp (Applied Photophysics Ltd., Surrey, England), operated with a bandwidth of ± 10 nm at the irradiation wavelength (350 nm or 480 nm). The intensity of the irradiation conditions was adjusted to 70 mW/cm^2 at the surface of the samples using a Thermophile voltmeter (Applied Photophysics Ltd.). Irradiation was performed in a quartz container under continuous stirring.

4.2.6. pH Measurements

pH was measured with a Microprocessor pH-mV-Meter, pH 526, from WTW GmbH, Weilheim, Germany.

4.2.7. Oxygen measurements

The concentration of oxygen in solutions was measured with a Dissolved Oxygen Hand-Held Meter, Oxi 340, and a Dissolved Oxygen Probe CellOx325, both from WTW GmbH, Weilheim, Germany.

4.3. Preparations

4.3.1. Thermal stability of the samples

Solutions containing epinephrine (2 μg/ml, sodium metabisulfite (1.82 μg/ml), sodium chloride (8.2 g/l) and sodium edetate (4.5 μg/ml) in potassium dihydrogene phosphate (5 mM, pH 4.7) were analysed stored in the dark at room temperature (n = 3). After 24 h no degradation of epinephrine was detected (4.2.1.). Samples (n = 12) containing epinephrine (2 μg/ml), sodium metabisulfite (10 μg/ml), sodium chloride (8.2 g/l) and sodium edetate (4.5 μg/ml) in potassium dihydrogene phosphate (5 mM, pH 4.7) were irradiated in the Suntest cps (4.2.4) for 7.1 h, analysed, stored for 24 h in the dark at room temperature and reanalysed (4.2.1.). No degradation was observed under the given conditions (0.2% ± 1.6%).

4.3.2. Influence of bisulfite and adrenochrome

Three samples (1 l) were prepared containing epinephrine (2.0 μg/ml, reference standard purity), sodium chloride (8.2 g/l) and sodium edetate (4.5 μg/ml) in potassium dihydrogene phosphate (5 mM, pH 4.7). To the solutions were added either sodium metabisulfite (1.8 μg/ml) or adrenochrome (1.0 μg/ml) or a mixture of both sodium metabisulfite (1.8 μg/ml) and adrenochrome (1.0 μg/ml). The solution with the exception of epinephrine and adrenochrome was prepared 15 h prior to irradiation. The latter components were added 30 min prior to irradiation. 10 ml samples (n = 3) and one control (wrapped in aluminium foil) were placed inn the Suntest CPS and irradiated for 7 h (4.2.4.). 250 μl of the solution was withdrawn and quantified at 60 min intervals (4.2.1.). pH of the solutions was determined after irradiation (4.2.6.).

4.3.3. Reaction of adrenochrome with bisulfite

A UV/Vis-absorption spectrum of a solution containing epinephrine (100 μg/ml) and adrenochrome (12 μg/ml) was recorded (4.2.2.). Sodium metabisulfite (3.7 μg/ml) was added and UV/Vis-absorption spectra were recorded at 15, 45, 90 and 120 min, and again after 20 h.

4.3.4. Influence of wavelength and oxygen

Four samples were prepared containing epinephrine (2.0 μg/ml), adrenochrome (1.0 μg/ml), sodium chloride (8.2 g/l) and sodium edetate (4.5 μg/ml) in potassium dihydrogene phosphate (5 mM, pH 4.7). Three of the solutions also contained sodium metabisulfite (1.8 μg/ml). The solution with the exception of epinephrine and adrenochrome was prepared 15 h prior to irradiation. The latter components were added 30 min prior to irradiation. The samples were exposed to monochromatic irradiation for 7 h (4.2.5). The solutions (50 ml) were stirred throughout the 7 h and were in equilibrium with air. A 1 ml sample was withdrawn and quantified by HPLC at 60 min intervals (4.2.1.). The samples containing bisulfite were irradiated at 350 ± 10 nm and at 480 ± 10 nm in equilibrium with air, and at 350 nm ± 10 nm while flushed with nitrogen. The sample without bisulfite was irradiated at 350 ± 10 nm. After irradiation the pH was determined in each sample (4.2.6.).

4.3.5. Influence of radical scavengers and quenchers

Samples containing epinephrine (2 μg/ml), adrenochrome (1 μg/ml), sodium metabisulfite (1.82 μg/ml), sodium chloride (8.2 g/l) and sodium edetate (4.5 μg/ml) were prepared in potassium dihydrogene phosphate (5 mM, pH 4.7). In addition they could contain t-butanol (0.4 μl/ml or 2 μl/m), d-mannitol (0.44 mg/ml or 2.18 mg/ml), superoxide dismutase (from bovine erythrocytes; 4.4 U/ml or 100 U/ml), sodium azid (0.65 mg/ml), DABCO (1.12 mg/ml) or 2,5-dimetylfuran (0.2 μl/ml). The solutions were made 15 h prior to irradiation. Adrenochrome, epinephrine and the scavenger/quencher were added 30 min before irradiation. The exposure time for samples irradiated at 350 ± 10 nm was 7 h. The samples were in equilibrium with air and under continuous stirring (4.2.5.). 1 ml of the sample was withdrawn and quantified with respect to epinephrine every 60 min (4.2.1.). Samples were also exposed to a continuous irradiation source (Suntest CPS, > 310 nm, 15 min) (4.2.4.). The pH was determined in all samples after irradiation.

4.3.6. Detection of singlet oxygen

Solutions of adrenochrome (4.0 μg/ml; 22.4 μM) and sodium metabisulfite (8.5 μg/ml; 44.8 μM) were prepared in the following media: destilled water, deuterium oxide and a solution containing sodium chloride (8.2 g/l) and sodium edetate (4.5 μg/ml) in potassium dihydrogene phosphate (5 mM, pH 4.7). UV-Vis spectra of the solutions were recorded and the absorbance at 350 nm was measured (4.2.2.). The samples were excited at 350 nm (±10 nm) and the luminescence was detected between 1100–1500 nm (4.2.3.). Samples of adrenochrome in deuterium oxide were also made containing 18.7 μM, 15.6 μM and 13.0 μM adrenochrome respectively, and 37.3 μM, 31.1 μM and 25.9 μM sodium metabisulfite respectively. UV-Vis spectra of the samples were recorded and the absorbance at 350 nm was measured. The solutions were exited at 350 nm (± 10 nm) and luminescence was detected between 1100–1500 nm. The

correlation between absorbance at 350 nm and luminescence at 1270 nm (as area under the peak) was calculated.
An excess molar amount of sodium metabisulfite (0.13 mM) and epinephrine (100.0 μg/ml (0.6 mM) or 200.0 μg/ml (1.1 mM)) was added to solutions of adrenochrome (12.0 μg/ml; 67.0 μM) in purified water. A sample without epinephrine was used as reference. The solutions were exited at 350 nm and luminescence was detected between 1100–1500 nm.

4.3.7. Monitoring of oxygen consumption

Three samples were made containing epinephrine 100 μg/ml (0,55 mM), adrenochrome 12 μg/ml (67.0 μM), sodium chloride (8.2 g/l) and sodium edetate (4.5 μg/ml) in potassium dihydrogen phosphate (5 mM, pH 4.7). The solutions contained different concentrations of bisulfite. Three solutions were made so that the concentrations of sodium metabisulfite could give 39.0 μM, 19.0 μM and 9.0 μM bisulfite respectively at maximum dissociation. The samples were stored in the dark for 15 hours prior to irradiation. The samples were then irradiated for 75 min at 350 ± 10 nm under continuous stirring (4.2.5.). The concentration of oxygen was measured every minute (4.2.7.). The experiment was performed in triplicate. After irradiation the content of epinephrine was determined by HPLC (4.2.1.) and the pH was measured (4.2.6.). The absorbance at 350 nm and 480 nm were recorded before and after irradiation (4.2.2.). The same parameters were recorded for the references stored in the dark.
Samples were also made containing different amounts of epinephrine (200 μg/ml, 50 μg/ml and 25 μg/ml) in addition to adrenochrome 12 μg/ml (67.0 μM), sodium chloride (8.2 g/l) and sodium edetate (4.5 μg/ml) in potassium dihydrogen phosphate (5 mM, pH 4.7). 25 ml were withdrawn from each solution and kept as dark references. Sodium metabisulfite was added in a concentration of 3.71 μg/ml (19.6 μM). The samples were stored in the dark for 15 hours prior to irradiation. Again, the samples were irradiated for 75 min at 350 ± 10 nm under continuous stirring (4.2.5.). The concentration of oxygen was measured every minute (4.2.7.). The experiment was performed in triplicate. After irradiation the content of epinephrine was determined by HPLC (4.2.1.) and the pH was measured (4.2.6.). The absorbance at 350 nm and 480 nm were recorded before and after irradiation (4.2.2.). The same parameters were recorded for the references stored in the dark.

4.3.8. The effect of protecting filter on the photostability of epinephrine

Samples were made in potassium dihydrogene phosphate (5 mM, pH 4.7) containing epinephrine (2 μg/ml), sodium chloride (8.2 g/l) and sodium edetate (4.5 μg/ml). Two of the four solutions contained sodium metabisulfite (1.82 μg/ml). The samples were transferred to quartz cuvettes and exposed in the Suntest CPS for 7.1 h (4.2.4.). One cuvette with bisulfite and one without bisulfite were covered with a filter (cut-off at 418 nm). The two remaining quvettes were wrapped in aluminum foil. The experiment was performed in duplicate. Samples (250 μl) were withdrawn after 2.9 h and 7.1 h and the amount of epinephrine was determined by HPLC (4.2.1.).

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