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AUTOXIDATION OF DRUGS: PREDICTION OF DEGRADATION IMPURITIES FROM RESULTS OF REACTION WITH RADICAL CHAIN INITIATORS (*)

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Summary — In the study of the degradation of drug substances by molecular oxygen, their specific reaction mechanisms must be taken into account. The rate-determining step is usually the reaction of the substrate with a radical chain initiator, which is often an unknown impurity. The reactivity and selectivity of autoxidation can be controlled better by using a radical chain initiator, such as AIBN, than by changing the temperature or the oxygen pressure. In this paper the products profiles of four pharmaceutical substances in a simple oxidation test with AIBN are compared with the results of long term natural stability tests or with already established stabilities.

Stress testing is the basis of all studies of the stability of a new drug substance. The first aim of this kind of investigation is to discover the chemical and physical factors that can affect the stability of the molecule adversely, in order to design stable formulations. The second aim is to obtain samples of the drug contaminated with all possible and significant degradation impurities, in order to validate the analytical methods for the long term stability studies and to isolate the main impurities. While standard experimental conditions for the study of accelerated and long term stability are defined in all the regulatory guidelines, the protocol for the reactivity study must be fit to the particular chemistry of the molecule being examined. For investigation of the hydrolytic pathway of degradation, the general protocol is to study the effect of acids or bases at elevated temperatures on the stability of aqueous solutions of the drug substance, because it is well known that hydrolysis reactions are catalyzed by acids and bases. Oxidation is a more complex reaction, and the pharmaceutical literature describes stress testing with various oxidizing agents, such as hydrogen peroxide, heavy metal ions, acids, bases, high oxygen pressure, high temperature and, in some instances, strong oxidants such as potassium permanganate and chromic anhydride. Very often this literature emphasizes the poor predictiveness of this kind of stress testing. One reason for this poor predictiveness is that the operating mechanisms of the oxidation with the above reagents are completely different from the radical chain mechanism of autoxidation. Long term, room temperature degradation of an organic chemical is better simulated by using a radical chain initiator to accelerate the rate-controlling step of autoxidation. Use of this approach in the reactivity study has been described in the recent pharmaceutical literature[1-4]. In this paper the experimental conditions for use of some radical chain initiators and the predictivity of this kind of reactivity test for four examples will be discussed.

In the electronic structure of molecular oxygen[5], the highest occupied molecular orbitals are two degener π^* orbitals in which there must be two electrons. The ground state, according to the Hund rule, is the state in which each of these two orbitals is occupied by one electron, and the spins are parallel: this is the triplet ground state ($^3\Sigma g$) of the atmospheric molecular oxygen. Triplet dioxygen can be excited, both chemically and photochemically, to the first excited state with spin multiplicity 0, the singlet state $^1\Delta g$, 22 kcal higher than the ground state[5]. The triplet ground state is the state of dioxygen involved in autoxidation. The reactivity of triplet dioxygen toward organic molecules can be summarized as follows.

Electron-rich molecules such as pyrroles[6], α,β-unsaturated enamines[7], carbanions[5], 9,10-cyclopentane-4a,4b-dihydrophenanthrene[8], strained cycloalkenes[9] and, under more drastic conditions, tertiary amines, sulfoxides, alkenes and alkynes[10] can react with oxygen in an electron-transfer reaction:

$$R^- + (^3\Sigma g)\ O_2 \rightarrow R\cdot + O_2^{-\cdot} \qquad \text{(eq. 1)}$$

In addition, triplet oxygen reacts very fast with organic radicals:

$$R\cdot + (^3\Sigma g)\ O_2 \rightarrow ROO\cdot \qquad \text{(eq. 2)}$$

and this reaction is very important in propagation of radical chains.

However the vast majority of organic molecules are in the singlet state, and their reaction with triplet dioxygen:

$$RH + (^3\Sigma g)\ O_2 \rightarrow ROOH \qquad \text{(eq. 3)}$$

is spin forbidden. For this reason, a great many organic molecules, in spite of the large negative value of the Gibbs free energy of oxidation, are kinetically inert

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toward triplet oxygen. In the latter case, that of a singlet organic molecule, inert toward triplet dioxygen, the well established mechanism of autoxidation at "normal" room temperature is depicted in equations 4-7:

$In^{\cdot} + RH \rightarrow InH + R^{\cdot}$	Initiation	(eq. 4)
$R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$		(eq. 5)
$ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot}$	Propagation	(eq. 6)
$2\ ROO^{\cdot} \rightarrow$ Inert products	Termination	(eq. 7)

The rate-determining step of the radical chain is the initiation reaction (eq. 4), whereas the propagation step (eq. 5) is very fast. This is why the oxidation rate does not depend on the oxygen partial pressure[11]. In this case it is absolutely useless to increase oxygen partial pressure to accelerate the oxidation: only the initiator $In^{\cdot}$, often a trace contaminant or impurity, controls the overall oxidation rate. To obtain reproducible oxidation, the concentration of the initiator must be controlled[12], for instance by adding known amounts of chemically defined radical chain initiators.

We could try to accelerate autoxidations just by increasing the temperature, exploiting the Arrhenius law. But in the complex mechanism depicted above, each step has its own activation energy, and the Arrhenius law can break down because the rate-determining step, and thus the mechanism, changes as temperature increases. Thermally labile compounds, such as hydroperoxides, do not only decompose at high temperature, but become efficient catalysts of the oxidation, giving kinetic profiles and chemical selectivity very different from those at room temperature[12].

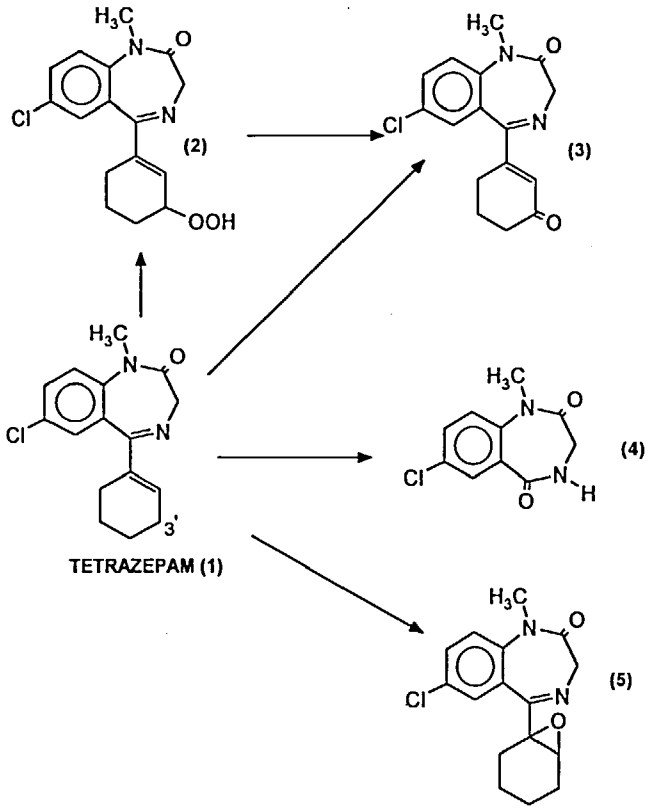
With combined high temperature and high oxygen pressure, the reaction path and the reaction products may change dramatically, because new mechanisms become effective. Amines and thioethers at ambient oxygen pressure and temperature undergo oxidation only in the α position to the heteroatom, often leading to a complex mixture of products. This a-oxidation fits with the mechanism of eqs. 4-7, corresponding to a reaction catalyzed by a foreign initiator. Under high oxygen pressure (70 bar) and at elevated temperature (100 °C), a specific oxidation of the heteroatom was obtained in high yields[10]. The mechanism of this oxidation involves the formation of an oxygen complex with the organic molecule, hence the need for high oxygen pressure, and a radical chain oxidation initiated by an electron transfer reaction.

RESULTS AND DISCUSSION

Tetrazepam

Tetrazepam 1 (Scheme 1) is a benzodiazepine used in therapy as myorelaxant. 1 is quite stable in solid pharmaceutical forms, but is oxygen-sensitive in solution. The main pattern of degradation of 1 (3) is the oxidation of the 3' carbon atom to give the 3'-

SCHEME 1



hydroperoxide 2 and the 3'-keto-derivative 3. Minor degradation impurities are the epoxide 5 and the product 4. We carried out an oxidation test with AIBN as a first approach to the profile of autoxidation impurities. An acetonitrile solution of the substance and of the radical chain initiator AIBN (2,2'-azobis[2-methylpropanenitrile]) was stored 48 h in the dark at 40 °C. The profile of degradation was very similar to that of a tablet sample in an accelerated stability study, i.e., after storage for 6 months at 55 °C and 85% relative humidity. Other stress tests (oxidation with Cu^{++} and Fe^{+++}, thermal stress of the bulk substance) did not produce the important degradation impurity 2. Reaction with hydrogen peroxide, widely used in preformulation studies, yielded only the minor impurity 5. Our conclusion was that, since AIBN oxidation occurs by the same radical chain mechanism as natural autoxidation, it is the best test to use to predict the oxidative behaviour[3].

The choice of temperature and solvent is very important in oxidative reactions. We never carry out the AIBN test on drug substances at a temperature over 40 °C, to prevent homolytic decomposition of species which are stable at ambient temperature, i.e., the hydroperoxide 2. Table I shows the solvent effects on the AIBN test. Acetonitrile is our preferred solvent because of its inertness to oxidants and its neutrality. Indeed, degradation of 1 is maximal in this solvent. Furthermore, replications of the test in acetonitrile gave

TABLE 1 - SOLVENT EFFECTS ON PRODUCT DISTRIBUTION FOR TETRAZEPAM (35 mM) AFTER DEGRADATION CATALYZED BY RADICAL CHAIN INITIATORS. (PERCENT COMPOSITION OF THE REACTION MIXTURE AFTER 48 h at 40°C)

Solvent	Catalyst	Composition (%)				
	(a)	1	2	3	4	5
Acetonitrile (b)	AIBN	65.8	8.4	11.2	1.9	2.8
Acetonitrile + water (20%) (b)	AIBN	68.1	9.6	11.2	1.9	2.8
Methanol	AIBN	75.2	8.3	6.6	2.4	1.8
Ethanol	AIBN	84.0	6.3	1.0	5.2	trace
2-Propanol	AIBN	89.6	4.3	0.3	4.8	trace
Acetonitrile + aq. buffer (c) 1:1	ACVA	89.4	3.3	5.0	1.3	1.0

(a) 60 mM. ACVA = 4,4'-azobis[4-cyanovaleric acid].
(b) From ref. 3.
(c) Phosphate buffer 0.022 M pH 7.0

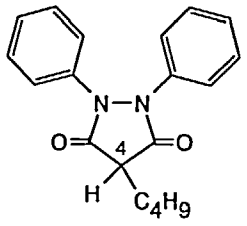
very reproducible results. The presence of water up to 20 % (V/V) has no important effect on the rate of degradation nor the distribution of the degradation product. Alcohols slow the reaction: in methanol the reaction is slightly slower than in acetonitrile, and in ethanol and isopropanol the oxidation is still slower. Alcohols probably inhibit oxidation by competing with the test substance for the initiator radicals. Indeed, we found acetone, the product of the solvent oxidation (0.2%) in an isopropanol solution of AIBN stored 48 h at 40 °C. Table I shows another important feature of the solvent effect in oxidation. The ratio between keto-impurity **3** and hydroperoxide impurity **2** decreases in parallel with the total degradation of **1**. Under neutral conditions and at moderate temperature, the hydroperoxide **2** is stable in solution. This means that the origin of the keto-impurity **3** is a reaction other than from decomposition of the hydroperoxide **2**, probably the termination reaction between two hydroperoxide radicals. According to this hypothesis, the most inhibitory solvent, isopropanol, is also the most powerful hydrogen-radical donor, which is able to efficiently quench the hydroperoxide radicals, decreasing formation of the keto- impurity.

AIBN is not soluble in water, and this may prevent using this reagent with very polar organic substances. Oxidation of tetrazepam with 4,4'azobis [4-cyanovaleric acid] at pH 7.0 in a 1:1 mixture of acetonitrile and water (Table I) gave qualitatively similar results. There was, however, a small difference in reactivity, seen as the formation of the minor impurity 3'-hydroxytetrazepam, an impurity never observed in the standard AIBN test.

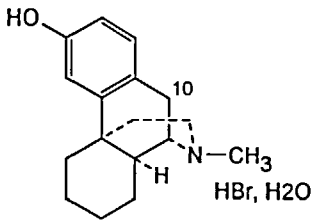
Phenylbutazone

To investigate the predictivity of the products of the oxidation with radical chain initiators, we carried out the test on drug substances with known degradation profiles and of different chemical classes, comparing the results with data in the literature.

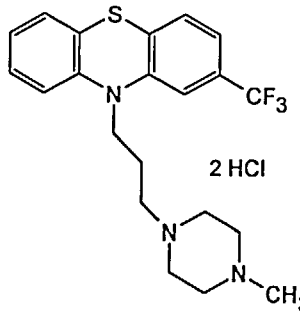
Phenylbutazone **6** (Fig. 1) is an oxygen-sensitive substance and oxydation yields 4-hydroxy-phenylbutazone **7**[14]. The AIBN test on **6** yielded **7** as the main product (6%) after 48 h at 40 °C and an unknown peak with an HPLC relative retention time of 0.78 (Fig. 2). After treatment with methionine (used as peroxide scavenger), the unknown peak disappeared, with an increase in the peak corresponding to **7**. The unknown peak probably corresponds to 4-hydroperoxy-phenylbutazone, which is the presumed but never isolated intermediate of **6** autoxidation. This example shows not only the predictivity of the results of reaction with the radical chain initiator, but also the possibility, because of its mild conditions, to accumulate labile intermediates.



PHENYLBUTAZONE (6)



DEXTROMETHORPHAN HYDROBROMIDE (8)



TRIFLUOPERAZINE DIHYDROCHLORIDE (10)

Fig. 1 - Structures of phenylbutazone, dextromethorphan hydrobromide and trifluoperazine dihydrochloride.

Dextromethorphan hydrobromide

Dextromethorphan hydrobromide **8** (Fig. 1) is a very stable drug substance. By photochemical oxidation, **8** yields 10-ketodextromethorphan **9**[15,16]. The same impurity was found in trace amounts during preformulation of an antitussive syrup containing **8**[17]. In the AIBN test, after 72 h at 40 °C, **8** underwent a slight degradation to give the impurity **9** (1 %). In the case of dextromethorphan, the low reactivity in the AIBN test reflects the good stability of the substance.

Trifluoperazine hydrochloride

Trifluoperazine dihydrochloride **10** (Fig. 1) is known to give the sulfoxide **11** by thermal and photochemical degradation[18]. Phenothiazines, and in particular **10**, are also known to directly react with oxygen by an electron-transfer mechanism. The AIBN test yielded

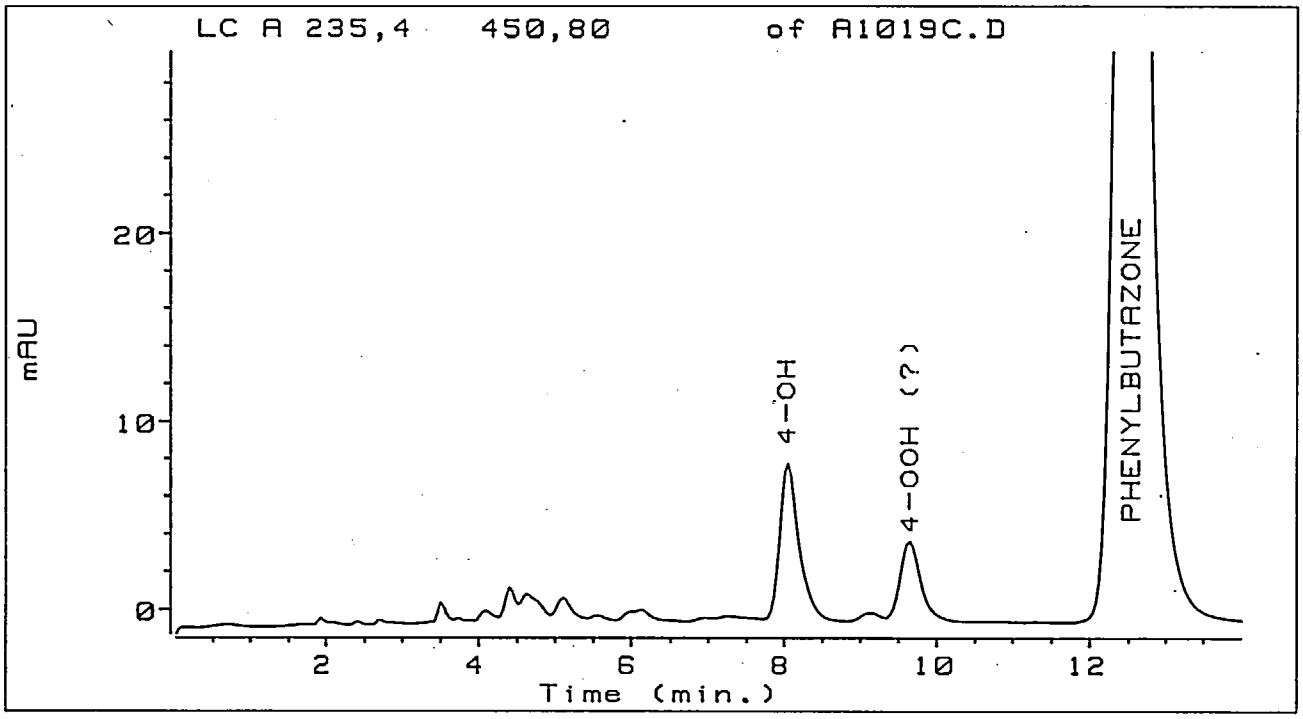


Fig. 2 - The HPLC chromatograms of phenylbutazone **6** in acetonitrile after 48-h degradation at 40°C in the presence of AIBN.

11 (67 %) as the only degradation product after 48 h at 40 °C. This result deserves some discussion. We expected **10** not to react in the AIBN test, or to give a different product, because AIBN acts as an hydrogen radical acceptor and the formation of the sulfoxide **11** must follow a different mechanism. Probably a peroxide, namely 2-hydroperoxy-2-methyl-propanenitrile and hydrogen peroxide, are products or intermediates of AIBN decompostion[19] and these oxidants can be the active species, via an ionic mechanism, in our AIBN test on **10**. Indeed, **10** reacts fast with hydrogen peroxide to give **11**. The ability of the AIBN test to predict the formation of the "good" degradation impurity is probably is due to a secondary reaction and not to a radical hydrogen extraction.

CONCLUSIONS

Oxidation by atmospheric dioxygen is a complex reaction, and its actual mechanism must be taken into account when carrying out stress tests to obtain the degradation impurities that might arise during long term storage of a drug and its pharmaceutical forms. Using a radical chain initiator such as AIBN or its soluble analogues is often a valid way to accelerate autoxidations.

EXPERIMENTAL PART

Materials and Reagents

Tetrazepam (Sanofi Chimie), phenylbutazone (CFM, Milan, Italy), dextromethorphan hydrobromide (Roche) and trifluoperazine dihydrochloride (ICM, Rozzano, Italy) were pharmaceutical grade compounds. **7**[14], **9**[15]and **11**[18] were prepared according to the references cited. AIBN (2,2'-azobis[2-methylpropanenitrile]) was obtained from Merck and ACVA (4,4'-azobis[4-cyanovaleric acid]) from Aldrich. All other chemicals were reagent or HPLC grade.

Instruments

The HPLC instrument consisted of a Perkin-Elmer series 3 pump, a Rheodyne 7161 injector with a 20-μl loop, a Hewlett Packard 1040 A diode array UV detector and a Hewlett Packard 79994A data station. The identity of each impurity in the chromatograms of the test solutions was confirmed by comparison of the retention time and of the UV spectrum at the peak maximum with the corresponding data obtained with an authentic sample of the impurity.

AIBN Test

The substance under examination was dissolved in acetonitrile or other solvent to give a solution about 10^{-2} M. A quantity of AIBN 1:1 w/w or mol/mol, as indicated in the individual experiments, was dissolved in the same solution. The solution was stored in a 25-ml pyrex vial fit with a screw cap, and the vial was stored in the dark at 40±0.5 °C. After the appropriate time, each solution was diluted with HPLC mobile phase to a suitable concentration and injected directly.

Tetrazepam AIBN Test

Tetrazepam (35 mM) and AIBN (60 mM) were dissolved in acetonitrile. HPLC analysis: column RoSil C18 HL (4.6 mm x 25 cm) 5 μm (R.S.L.); analytical wavelength 254 nm; mobile phase a mixture of 50 vol. of a 0.01 M aqueous potassium dihydrogen phosphate (pH 4.65) and 50 vol. acetonitrile; flow rate 1.1 ml.

Phenylbutazone AIBN Test

Phenylbutazone (32 mM) and AIBN (60 mM) were dissolved in acetonitrile. HPLC analysis: column μBondapack C18 (Waters) 30

cm x 3.9 mm; analytical wavelength 235 nm; mobile phase a mixture of 50 vol. of 0.1M phosphate buffer (pH 3.7) and 50 vol. acetonitrile; flow rate 1.2 ml/min.

Dextromethorphan hydrobromide AIBN Test

Dextromethorphan hydrobromide (14 mM) and AIBN (30 mM) were dissolved in the desired solvent (see table I). HPLC analysis: column μBondapack C18 (Waters) 30 cm x 3.9 mm; analytical wavelength 280 nm. Mobile phase: dioctyl sulfosuccinate (2.9 g) in 680 ml methanol and 290 ml water, with phosphoric acid (1 ml) added and pH adjusted to 3.8 with diluted ammonia; flow rate 1.3 ml/min.

Trifluoperazine dihydrochloride AIBN Test

Trifluoperazine dihydrochloride (21 mM) and AIBN (21 mM) were dissolved in acetonitrile. HPLC analysis: column Bondapack C18 (Waters) 30 cm x 3.9 mm; analytical wavelength 278 nm; mobile phase a mixture of 20 vol. of 0.006 M aqueous sodium hexanesulfonate (pH 3.8) and 80 vol. methanol.

REFERENCES

(1) G. Boccardi, G. Palmisano and G. Riva, *4° Convegno Nazionale di Chimica Farmaceutica*, Milan, 1988, poster.

(2) A.R. Oyler, R.E. Naldi, K.L. Facchine, D.J. Burinsky, M.H. Cozine, R. Dunphy, J.D. Alves-Santana, M.L. Cotter, *Tetrahedron, 47*, 6549 (1991).

(3) G. Boccardi, C. Deleuze, M. Gachon, G. Palmisano and J.P. Vergnaud, *J. Pharm Sci., 81*, 183 (1992).

(4) G.B. Smith, L. DiMichele, L.F. Colwell, G.C. Dezeny, A.W. Douglas, R.A. Reamer, T.R. Verhoeven, *Tetrahedron, 49*, 4447 (1993).

(5) H. Knopf, E. Mueller, A. Weickmann, in: Houben-Weyl Methoden der Organischen Chemie, vol. 4, part 1a, G.Thieme, Stuttgart, 1981, p.69.

(6) B.D. Beaver, J.V. Cooney, J.M. Jr Watkins J.M., *Heterocycles, 23*, 2847 (1985).

(7) S.K. Malhotra, J.J. Hostynek and A.F. Lundin, *J. Am. Chem. Soc., 90*, 6565 (1968).

(8) A. Bromberg, K.A. Muszkat, *J. Am. Chem. Soc., 91*, 2860 (1969).

(9) P.D. Bartlett, R. Banavali, *J.Org. Chem., 56*, 6043 (1991).

(10) P. Correa, G. Hardy, D.P. Riley, *J.Org. Chem., 53*, 1695 (1988).

(11) R.A. Sheldon, J.K. Kochi, Metal-catalyzed Oxidations of Organic Compounds, Academic Press, New York, 1981, p. 18.

(12) G.W. Burton and K.U. Ingold, *J. Am. Chem. Soc., 103*, 6472 (1981).

(13) K.A. Connors, G.L. Amidon and V.J. Stella, Chemical Stability of Pharmaceuticals, 2nd ed., Wiley, New York, 1986, p. 92.

(14) D.V.C. Awang, A. Vincent, F. Matsui, *J. Pharm Sci., 62*, 1673 (1973).

(15) Q. Haefiger, A. Brossi, L.H. Chopard-dit-Jean, Q. Walter, Q. Schnider, *Helv. Chim Acta, 39*, 2953 (1956).

(16) G. Boccardi, P. Mezzanzanica, U. Guzzi, G. Lesma, G. Palmisano, *Chem. Pharm Bull., 37*, 308 (1989).

(17) G. Boccardi, unpublished results.

(18) Analytical Profile of Drug substances, Florey K., Ed., Academic Press, San Diego, CA, vol. 9, 1980, p. 543.

(19) A. Goosen, C.W. McCleland, D.H. Morgan, J.S. O'Connel, A. Ramplin, *J. Chem. Soc. Perkin Trans. I*, 1993, 401.

NOTE ADDED IN PROOF - We became aware of an error in the paragraph "Phenylbutazone". The synthesis and spectroscopic characterisation of 4-hydroperoxy-phenylbutazone has been previously reported by Von P. Menz, M. Schulz and R. Kuge, *Arzneim. - Forsch./Drug Res. 37(II)*, 1229 (1987). This compound was also detected as a major degradation product of phenylbutazone in a sample of 4-years old tablets.