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Measurement of β-agonist residues in retinal tissue of food producing animals

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This report presents the results of an investigation on the accumulation of β-agonist residues in the retinal tissue of food producing animals. Three different species, calf, pig and turkey, were treated with six different β-agonists and analysed for β-agonist residues in retinal tissue applying a newly developed retina preparation procedure which provides sufficient sample material for multiple analyses. The results show that all selected β-agonists accumulate in the retina, though in varying concentrations. The results are discussed on the basis of existing binding theories and with regard to their impact on the existing residue control strategy for β-agonists.

Introduction

β-adrenergic drugs (β-agonists) are therapeutically used in human and veterinary medicine for the treatment of bronchoconstrictions. Since the late 1980s a widespread abuse of β-agonists, mostly in the form of clenbuterol-containing black market products, in food producing animals for fattening became evident not only in the EU but also in many other countries.[1] In order to protect consumers from exposure to unacceptable residue concentrations in food products of animal origin, an efficient control is required. Previous investigations showed a persisting accumulation of the β-agonist clenbuterol in retinal tissue.[2] Moreover, several other drugs are known to accumulate in pigmented tissue.[3–6] Concerning clenbuterol, experiments have shown that the concentrations in the retina exceeded those in liver by at least two orders of magnitude and the elimination half-life is approximately five times higher than in liver.[2,7–9] Taking into account that live animal sampling is generally restricted to blood, urine and faeces, better options are provided by slaughter where whole-eye sampling becomes easily available. Thus, for veterinary drugs tending to accumulate in pigmented tissue, there may be many potential advantages in pigmented tissue analysis within surveillance programs.

In vitro studies demonstrated a high potential for melanin–drug interactions,[7–14] which are only rarely demonstrated in living animals.[7] These interactions are of different natures like electrostatic forces occurring between positively charged drug molecules and negative groups of the melanin polymer or van der Waals forces at the conjugations of the aromatic rings in the substances and the aromatic indol nuclei of melanin. A further interaction could be based on charge transfer complexes assuming that drugs which are good electron donors would be able to participate in such a complex with the help of the free radical of melanin.[11–13] At physiological pH many drugs with known melanin-affinity are present as cations so that electrostatic forces seem to be involved more generally in these interactions.[13] Furthermore, the lipophilicity of the drugs seems to be of importance in view of their accumulation in pigmented tissues. It could be shown that the high liposolubility of nonphenolic amines might be responsible for their greater accumulation in pigmented iris.[11,14]

β-Agonists can be roughly divided into two chemical groups: the clenbuterol-like substances with anilinic moieties and the salbutamol-like drugs with phenolic, catecholic or resorcinolic moieties; owing to their misuse potential in food producing animals, this substance group is of high interest for further investigations. All the more so as according to our knowledge, no comprehensive study has been carried out so far using various drugs in living food producing animals. As a first step in the present study the clenbuterol-like substance group was investigated. Therefore, clenbuterol, brombuterol, clenproperol, propranolol, mabuterol and cimaterol (Fig. 1) were applied to three different species each, namely to calves, pigs and turkeys. The results are given and discussed on the basis of existing binding theories. Consequences for sampling strategies and surveillance programs are discussed.

A retina preparation procedure is described which allows a loss-free production of retina suspensions for multiple analyses.

Experimental

Reagents and materials

Unless indicated otherwise, only analytically pure chemicals were used; all organic solvents were in line with the purity requirements 'for residue analysis'. d_9-Clenbuterol (internal standard; IS) and clenproperol were purchased from Witega (Berlin-Adlershof, Germany). d_7-Cimaterol and d_9-mabuterol were provided by RIVM (Bilthoven, The Netherlands) and mabuterol, clenbuterol and cimaterol were supplied by Boehringer (Ingelheim, Germany). Brombuterol was purchased from the Université de Rennes (Rennes, France). Propranolol was obtained from Sigma (Deisenhofen, Germany). Ethyl acetate, hexane, tertiary butyl methyl ether, ethanol, tris(hydroxymethyl)aminomethane, calcium chloride dihydrate, 0.2 M sulfuric acid, sodium hydroxide, molecular sieve 0.3 nm, and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Protease pronase E, Typ XIV and methane boronic acid (97%) (MBA) were purchased from Sigma. The deionized water was obtained from Fisher Scientific (Wiesbaden, Germany).

The 0.2 M TRIS HCl buffer was produced by weighing 24.2 g of tris(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$) and 14.7 g of calcium chloride ($CaCl_2 \cdot 2\ H_2O$) and then making up to 1 l

with deionized water. The pH was adjusted to 8.0 by using 1 M HCl. The methaneboronic acid solution, which was prepared freshly for every series of samples, was produced by dissolving 1 mg of methaneboronic acid in 1 ml of dried ethyl acetate (molecular sieve, 0.3 nm). The protease solution was produced freshly for every run by diluting 50 mg of protease in 1 ml of deionized water. The dilution of the analytical standards was carried out with ethanol.

Equipment and GC-MS conditions

A Hewlett-Packard 5890 Series II Plus gas chromatograph with a mass-selective detector HP 5972 and an automatic sampler HP 7673 A (Waldbronn, Germany), was used. The gas chromatograph was equipped with a split–splitless injector. The measurements were carried out using the electron impact ionisation mode (EI). A DB5 methyl–5% phenylsilicone column of 30 m length and of an internal diameter of 0.25 mm was used. The film thickness was 0.25 μm. The injection volume was 2 μl. The injection block and the interface were heated to 280 °C. The oven temperature programme started at 120 °C for 0.1 min followed by a gradient of 15 °C min^{-1} to 300 °C (held for 5 min).

For the homogenisation of the retinal tissue a potter homogeniser from B. Braun Biotech International (Melsungen, Germany) was used.

Procedure

Retina preparation. The preparation of the retina was carried out as follows: after dissecting the retina from the eye, the whole material was weighed into a ground centrifuge tube (80 ml). The weight of the retina was recorded exactly. Then the laboratory sample was mixed with TRIS HCl buffer to exactly 11 g total weight for calves and 6 g total weight for pigs or turkeys. After homogenising the retina in the potter for approx. 10 min, the laboratory sample was divided into 3–10 (depending on the volume available) sub-samples of 1 g each by transferring them into 12 ml plastic tubes with the help of a measuring pipette. The sub-samples were stored in a freezer at −25 °C.

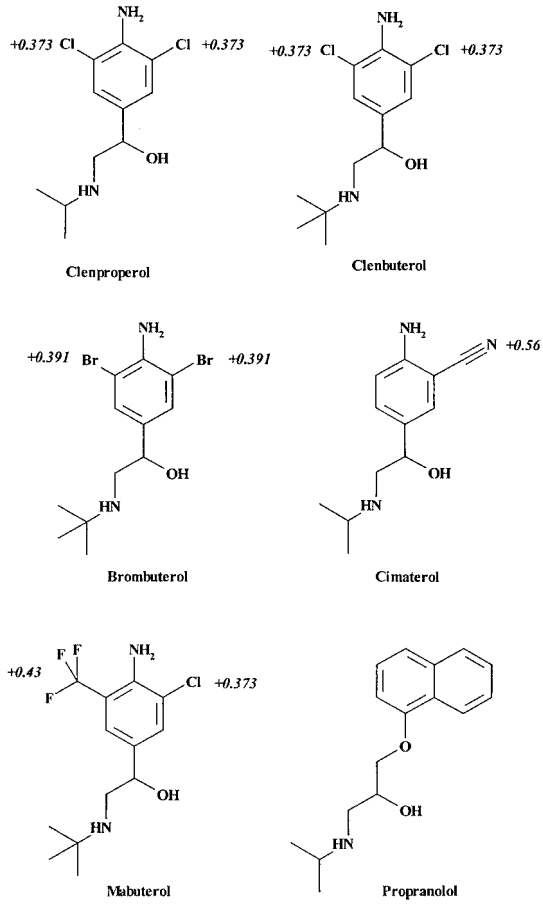

Fig. 1 Chemical structures of the applied drugs. Figures given in italic are the substituent factors.[28]

Sample preparation and clean-up. The sub-samples were transferred into 80 ml ground centrifuge tubes. After again adding buffer solution (0.2 M TRIS HCl buffer) to an amount of exactly 10 g, they were homogenised using an ultrasonic rod. Afterwards the homogenised sub-samples were aliquotted in a way appropriate for use as test samples. The initial sample quantity of this procedure was 3 ml of diluted retina suspension. The internal standard solution was added directly after the homogenisation and aliquotting of the retina samples. The following procedure for the sample clean up was carried out according to Blanchflower *et al.*,[16] introducing only minor modifications.

Confirmatory analysis

According to Commission Decision 93/256/EEC, for confirmatory purposes four diagnostic ions of the MBA derivatives of the β-agonist(s) were measured (Table 1).

Limit of detection and limit of determination

The limit of detection (LOD) and the limit of determination (LOQ) were calculated according to DIN 32645-1994[15] with an α-error probability of 0.05 on the basis of a matrix calibration curve calculated for 40 mg retina. The calculated limits per analyte, expressed in concentration units and the corresponding absolute amounts, are listed in Table 2.

Table 1 Diagnostic ions monitored in the EI mode

Substance	Relative retention time	Base peak (*m*/*z*)	Fragment ion 1 (*m*/*z*)	Fragment ion 2 (*m*/*z*)	Fragment ion 3 (*m*/*z*)
d_9-Mabuterol (IS)	1.000	283	—	—	—
Mabuterol	1.006	277	319	279	334
d_9-Clenbuterol (IS)	1.000	249	—	—	—
Clenproperol	0.931	229	231	271	286
d_7-Cimaterol (IS)	1.000	190	—	—	—
Cimaterol	1.004	186	228	243	144
d_9-Clenbuterol (IS)	1.000	249	—	—	—
Clenbuterol	1.002	243	245	285	300
Clenbuterol (IS)	1.000	243	—	—	—
Propranolol	1.037	128	283	268	140
d_9-Clenbuterol (IS)	1.000	249	—	—	—
Brombuterol	1.114	333	375	335	390

Table 2 Limits of detection (LOD) and determination (LOQ) of the applied substances

Substance	LOD/μg kg^{-1}	LOD/ng	LOQ/μg kg^{-1}	LOQ/ng
Mabuterol	117	4.68	172	6.88
Clenproperol	138	5.52	205	8.20
Cimaterol	117	4.68	174	6.96
Clenbuterol	110	4.40	164	6.56
Brombuterol	105	4.20	155	6.20
Propranolol	240	9.6	350	14.0

Precision and recovery

The within-laboratory reproducibility s_{wR} was determined analysing three different runs with fortified blank matrices of different species within five weeks (Table 3). The recovery R was determined using fortified blank bovine retina (Table 3).

Treatment of animals

Five calves (Deutsche Schwarzbunte) and five pigs (Deutsches Sattelschwein) were treated according to schemes described in the literature.[17–25] Since so far a veterinary application scheme is not yet available, the dosages recommended in human medicine by the so-called 'Rote Liste'[26] were adapted to the respective body weights of the animals. The total amounts of pure drugs were diluted in 700 ml of water so that 50 ml per day could be applied during a treatment period of 14 days. The calves were fed twice a day approximately 8 l of milk replacer in such a way that the 50 ml of drug solution were mixed with a small amount of the milk replacer and administered as a first feeding portion to the animals, in order to ensure complete reception of the drug. Only then was the remaining milk replacer fed as a second portion. Additionally, 125 g of structure cobs per day were fed. The pigs were fed twice a day 1 kg of fodder suspension each. Here again, at first a small part of the fodder suspension was mixed with 50 ml of drug solution to ensure complete ingestion of the drug, and afterwards the rest of the fodder was given.

Six turkeys (BUT-Big6) were treated with cimaterol as described in literature.[27,28] Most of the substances were not authorised for poultry or animal treatment. In these cases dosages like those applied for the pigs were adapted. The required dosage was calculated by taking into account the individual body weight and the average drinking amount of each turkey. The total amount of pure drugs was diluted in 700 ml of water. A 50 ml portion of this solution per day was diluted again with 1950 ml of water. This quantity of 2 l was offered to each turkey and the actual daily volume drunk was measured in order to calculate the exact amount of drug ingested. Detailed information on feeding schemes is given in Table 4.

Table 3 Within-laboratory reproducibility (s_{wR}) and recovery (R) of the applied substances

Substance	s_{wR} (%) at 100 μg kg^{-1} ≡ 4 ng	s_{wR} (%) at 500 μg kg^{-1} ≡ 20 ng	R (%) at 100 μg kg^{-1} ≡ 4 ng	R (%) at 500 μg kg^{-1} ≡ 20 ng
Mabuterol	3.2	6.9	103	91
Clenproperol	—[a]	26.7	—[a]	98
Cimaterol	18	7.5	95	96
Clenbuterol	6.1	7.8	97	97
Brombuterol	4.4	6.7	105	104
Propranolol	—[a]	7.58 (at 30 ng)	—[a]	87 (at 30 ng)

[a] Not available at this concentration.

Results and discussion

The results presented are based on single animal treatment. For this reason the inter-animal variability has not been taken into consideration, and consequently both the species- and the substance-specific differences must be discussed with respect to this background.

In the experiment presented, all investigated substances accumulated in retina tissues, although varying widely in concentration levels.

In calves and pigs, brombuterol and clenbuterol yielded very similar residue levels of approximately 2000 μg kg^{-1}; this also applies to turkeys but at a lower level, approx. 200 to 600 μg kg^{-1} (Table 5). This is in good agreement with the substituent constants† as given in Fig. 1, according to which bromine and chlorine groups have almost the same inductive effect.[28]‡ Compared with clenbuterol and brombuterol, clenproperol and mabuterol produce lower residue concentrations (approx. 300 μk kg^{-1}) in the species investigated. This is a very interesting result, in so far as clenproperol differs from clenbuterol only in

† Substituent-specific constant which reflects the effect of the substituent on the parent substance of the molecule (*e.g.*, benzene).[29]

‡ Concept developed mainly by Lewis and Ingold aimed at investigating the influence on reactivity of electropositive and electronegative substituents in organic molecules. Information on the strength of the inductive effect is provided by dipole moments.[30]

Table 4 Treatment scheme for calves, pigs and turkeys

Animal	Substance applied	Body weight/kg	Days of treatment	Amount of drug applied per day/mg	Actual dose/ mg kg^{-1} of body weight	Total amount of applied drug/mg
Calves—						
C1	Brombuterol	143	15	1.144	0.008	17.16
C2	Clenproperol	147	15	1.176	0.008	17.64
C3	Propranolol	126	15	189	1.500	2835
C4	Mabuterol	156	8	1.248	0.008	9.984
C5	Cimaterol	147	13	7.35	0.050	95.55
C6	Clenbuterol	Not administered				
Pigs—						
P1	Brombuterol	26	14	0.416	0.016	5.824
P2	Propranolol	18	14	27.00	1.500	378.03
P3	Mabuterol	23	14	0.368	0.016	5.152
P4	Cimaterol	106.5	13	5.325	0.050	69.225
P5	Clenbuterol	25	14	0.400	0.016	5.600
P6	Clenproperol	Not administered				
Turkeys—						
T1	Brombuterol	4.15	12	0.0296	0.00712	0.355
T2	Clenproperol	4.20	12	0.0367	0.00873	0.440
T3	Propranolol	3.50	12	5.638	1.611	67.65
T4	Mabuterol	4.36	12	0.0469	0.0108	0.562
T5	Cimaterol	3.46	12	0.700	0.202	8.40
T6	Clenbuterol	4.26	12	0.0451	0.0106	0.542

one methyl group at the ethanolamine moiety, and mabuterol differs from this compound in one trifluoromethyl group at the phenyl ring. This might lead to the conclusion that not only one binding mechanism is involved in the substance–melanin interaction, but at least, on the basis of provisionally considering ionic interactions only, both amine groups are involved, the anilinic as well as the ethanolamine group. CF_3 groups have a −I-effect (negative inductive effect) affecting the basicity of the amine in the *ortho*-position in such a way that it shows lower basic properties and therefore a lower proton affinity than clenbuterol for example. These effects are described in other publications[27] where substituent constants are calculated, as listed in Fig. 1. On the other hand methyl groups have +I-effects (positive inductive effect) increasing the basicity of amines. Both effects might lead to less protonated mabuterol and clenproperol compared to clenbuterol and brombuterol at physiological pH and consequently to a lower binding tendency to the carboxy groups of the melanin polymers. These very theoretical considerations should be subjected to further investigations.

Cimaterol was found in a very high concentration in turkey retina (approx. 3000 μg kg^{-1}) whereas it was present in calf and pig retinas in much lower concentrations of about 600 μg kg^{-1}, which might be due to the higher dose of drug that had been administered to the turkeys.

Propranolol, although not a clenbuterol-type β-agonist, accumulated in retinal tissue in a concentration range similar to clenproperol and mabuterol.

The binding of the investigated substances to melanin might be due to ionic interactions as discussed, but also to conjunctions of aromatic rings of the analytes and the melanin polymer. Finally it might be due to the formation of bridge bindings *via* phenolic OH functional groups, which are also present in the melanin.[13]

These considerations must be seen in relative terms when considering not only physico-chemical properties in the retina itself but also pharmacological aspects like administration schemes, bioavailability and metabolic pathways. Therefore it has to be taken into account that actually all drugs should be administered in therapeutic dosages. Since brombuterol is not authorised, no application scheme was available. Referring to its structural analogy to clenbuterol the dosages were adapted appropriately. For the treatment of turkeys no application schemes were available either, except for cimaterol, therefore they were treated in analogy to the pigs. These insufficiencies might also cause variations in the accumulation pattern. Furthermore, since all substances were applied as pure substances and not as drug formulations, the bioavailability was certainly not optimised and therefore probably caused differences in accumulation, too. Hence, at first glance, no dependency of the total amount of drug administered and the detected residue concentrations seems to exist (Table 5).

Table 5 Residue levels of β-agonists in calves, pigs and turkeys

Substance applied	Residues in retina after 0 days of withdrawal time[a]/μg kg^{-1}		
	Calves	Pigs	Turkeys
Brombuterol	2059	2170 ± 192	186 ± 7
Clenproperol	337 ± 30	—	283 ± 1
Propranolol	361.14 ± 0.46	18.70 ± 0.27	401 ± 10
Mabuterol	510 ± 66	158 ± 6	232 ± 6
Cimaterol	633 ± 13	724 ± 166	3223 ± 63
Clenbuterol[b]	—	2039 ± 10	607 ± 12

[a] The given standard deviations are repeatability standard deviations calculated on the basis of two parallel analyses. [b] From clenbuterol applied in therapeutic dosages to veal calves, which are then slaughtered without any withdrawal time, it is known that residues of approximately 2000 μg kg^{-1} in retina can be found.[6]

The standard deviations of analyses in duplicate of the individual samples showed a very good repeatability with RSDs of between 0.5 and 13%. The only outlier is the repeatability of cimaterol in pigs with a RSD of 23% (Table 5). The results presented prove the high homogeneity of the produced retina suspensions and the usefulness of the described procedure for the production of retina suspensions for residue surveillance purposes. The procedure described above allows the production of up to 20 sub-samples on the basis of the retina of the two eyes of one animal. Consequently, the so far existing limitations of sufficient sample material to repeat retina analyses have been overcome. Applying the described approach to the preparation of retina samples for counter analyses and arbitration analyses with respect to official residue controls can be carried out easily and reliably.

Conclusion and outlook

Investigations of clenbuterol in retina[2,8,9] had revealed the very high accumulation potential of this tissue combined with a slow depletion behaviour of the substance. For this reason retina is a matrix of great interest for residue control purposes for this β-agonist.

It was demonstrated in this study that beside clenbuterol also brombuterol, clenproperol, mabuterol, cimaterol and propranolol accumulate in the retina of calves, pigs and turkeys. The results so far achieved encourage the combination of these investigations, studying the inter-animal variability and the depletion behaviour of β-agonists in retina in order to provide more powerful tools for a reliable and effective control of residues in animal products intended for human consumption.

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References

1 S. Hahnau and B. Jülicher, *Food Addit. Contam.*, 1996, **13**(3), 259.
2 I. Schmädicke, PhD Thesis, BgVV, Diedersdorfer Weg 1, 12277, Berlin, Germany, 1999.
3 M. M. Salazar-Bookaman, I. Wainer and P. N. Patil, *J. Ocul. Pharm*, 1994, **10**(1), 217.
4 C. G. Mason, *J. Toxicol. Environ. Health*, 1977, **2**, 977.
5 M. Araie, M. Takase, Y. Sakai, Y. Ishii, Y. Yokoyama and M. Kitagawa, *Jpn. Ophthalmol.*, 1982, **26**, 248.
6 R. E. Joseph, Jr., T.-P. Su and E. J. Cone, *J. Anal. Toxicol.*, 1996, **20**, 338.
7 J. M. J. Sauer and S. P. L. Anderson, *Analyst*, 1994, **119**, 2553.
8 C. T. Elliot, S. R. H. Crooks, J. G. D. McEvoy, W. J. McCaughy, S. A. Hewitt, D. Patterson and D. Kilpatrick, *Vet. Res. Commun.*, 1993, **17**, 59.
9 C. T. Elliott, W. J. McCaughy, S. R. H. Crooks, J. D. G. McEvoy and D. G. Kennedy, *Vet. Q.*, 1995, **17**(3), 100.
10 L. Howells, M. Godfrey and M. J. Sauer, *Analyst*, 1994, **119**, 2691.
11 K. Shimada, R. Baweja, T. Sokoloski and P. N. Patil, *J. Pharm. Sci.*, 1976, **65**(7), 1057.
12 H. Tjälve, M. Nilsson and B. Larsson, *Biochem. Pharm.*, 1981, **30**(13), 1845.
13 B. Larsson and H. Tjälve, *Biochem. Pharm.*, 1979, **28**, 1181.
14 R. Kaliszan, A. Kaliszan and I. W. Wainer, *J. Chromatogr.*, 1993, **615**, 281.

15 Deutsches Institut für Normung, DIN 32645, *Chemical Analysis, Decision Limit, Detection Limit and Determination Limit; Estimation in Case of Repeatability, Terms, Methods, Evaluation*, Beuth Verlag, Berlin, 1994.
16 W. J. Blanchflower, A. Hewitt A. Cannavan, C. Elliott and A. Kennedy, *Biol. Mass Spectrom.*, 1993, **22**, 320.
17 W. Löscher, F. R. Ungemach and R. Kroker, *Grundlagen der Pharmakotherapie bei Hausund Nutztieren*, Verlag Paul Parey, Berlin, Hamburg, 2nd edn., 1994, pp. 53–59.
18 J. Salobir, *Landbauforschung Völkenrode*, Wissenschaftliche Mitteilungen der Bundesanstalt für Landwirschaft Braunschweig Völkenrode (FAL), Sonderheft 114, 1990, pp. 25–31.
19 N. H. Williams, T. R. Cline, A. P. Schinckel and D. J. Jones, *J. Anim. Sci.*, 1994, **72**(12), 3152.
20 M. E. Spurlock, J. C. Cusumano, S. Q. Ji, D. B. Anderson, C. K. Smith, D. L. Hancock and S. E. Mills, *J. Anim. Sci.*, 1994, **72**(1), 75.
21 J. Salobir, M. Henning, E. Farries and E. Kallweit, *Fleischwirtschaft*, 1990, **70**(12), 1472.
22 C. Y. Liu, A. L. Grant, K. H. Sim, S. Q. Ji, D. L. Hancock, D. B. Anderson and S. E. Mills, *J. Anim. Sci.*, 1994, **72**(1), 62.
23 D. J. A. Cole, J. D. Wood and M. J. Kilpatrick, in *Beta-Agonists and their Effects on Animal Growth and Carcass Quality*, ed. J. P. Hanrahan, Elsevier Applied Science, London, 1987, pp. 137–142.
24 F. R. Dunshea, *Pig News Inform.*, 1991, **12**(2), 227.
25 C. J. M. Arts, M. J. van Baak and J. van der Greef, Poster Presentation, International Symposium on Hormone and Veterinary Drug Analysis, Bruges, 1994.
26 Rote Liste, Rote Liste® Service GmbH, Frankfurt/Main, ECV Editio Contor Verlag für Medizin und Naturwissenschaften GmbH, Aulendorf/Württ., Germany, 1998.
27 K. L. Rogers and J. M. Fagan, *Proc. Soc. Exp. Biol. Med.*, 1991, **197**(4), 482.
28 W. F. Dean and R. H. Dalrymple, *Poultry Sci.*, 1988, **67**, (Suppl. 1), 73A.
29 D. H. McDaniel and H. C. Brown, *J. Org. Chem.*, 1958, **23**, 420.
30 *Römpp Chemie Lexikon*, ed. J. Falbe and M. Regitz, Thieme Verlag Stuttgart, New York, 9th edn., 1995, p. 1973.