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## Note

# Analysis of epoxyeicosatrienoic acids by gas chromatography—mass spectrometry using chlorohydrin adducts

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Oxygenation of arachidonic acid to epoxides seems to play a pivotal role in eicosanoid biology. An allylic epoxide, leukotriene $A_4$, is formed via the 5-lipoxygenase pathway and it can be converted to leukotriene $B_4$ or to the peptidoleukotrienes, which possess potent biological activity [1]. Four epoxyeicosatrienoic acids are formed from arachidonic acid by cytochrome P-450 in the liver and the renal cortex [2—6]. Recent studies show that some of these epoxyeicosatrienoic acids also exert biological effects [7—9] and one of them, 5(6)-epoxy-20:3, is the precursor of a series of novel prostaglandins [10—12].

The epoxyeicosatrienoic acids are hydrolysed to vicinal diols by epoxide hydrolases [4, 13], and methods for analysis of these vicinal diols by mass fragmentography have been described [6, 14]. The epoxides have also been isolated and identified by gas chromatography—mass spectrometry (GC—MS) [4, 5], but the mass spectra of the four methyl epoxyeicosatrienoates provide limited structural information and appear to be unsuitable for mass fragmentography. Trapping of epoxides by conversion to chlorohydrin adducts by treatment with trimethylchlorosilane (TMCS) to facilitate identification of epoxides was introduced by Harvey et al. [15]. In view of the recent interest in the formation of epoxyeicosatrienoic acids from arachidonic acid, the chlorohydrin adducts of these epoxides were synthesized and analysed by GC—MS. The possible use of the chlorohydrin adducts of octadeuterated 5(6)-epoxy-20:3 as internal standards for mass fragmentography was also evaluated.

## MATERIALS AND METHODS

Arachidonic acid (99%) was from Sigma (St. Louis, MO, U.S.A.). [$^3H_8$]-

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arachidonic acid (131 Ci/mmol) was purchased from The Radiochemical Centre (Amersham, U.K.), while [5,6,8,9,11,12,14,15-$^2H_8$]arachidonic acid, which contained less than 0.5% of the protium form, was a generous gift from Dr. D.A. van Dorp (Unilever Research, Vlaardingen, The Netherlands). The isotope content was $^2H_{10}$ 3%, $^2H_9$ 16%, $^2H_8$ 59%, $^2H_7$ 19% and $^2H_6$ 3% as judged from GC—MS analysis of the methyl ester. The following epoxides, 14(15)-epoxy-20:3, 11(12)-epoxy-20:3, 8(9)-epoxy-20:3 and 5(6)-epoxy-20:3, were synthesized and characterized as described previously [4, 6, 14]. Octadeuterated 5(6)-epoxy-20:3 was synthesized from 1.9 mg of deuterated arachidonic acid and 180 μCi of [$^3$H]arachidonic acid as described previously [6, 16, 17]. Bis(trimethylsilyl)trifluoracetamide (BSTFA) was from Supelco (Bellefonte, PA, U.S.A.). Glass plates, precoated with 0.25-mm silica gel 60 F254 (10 × 20 or 5 × 10 cm) for thin-layer chromatography (TLC), TMCS and most other chemicals were from E. Merck (Darmstadt, F.R.G.).

An ethereal solution of diazomethane was used for methylation. Chlorohydrin adducts were obtained by treatment with 1.2 *M* hydrochloric acid in pyridine (20 μl) for 1 h at 65°C. After evaporation under a stream of nitrogen, the residue was purified by TLC (as methyl esters; hexane—ethyl acetate, 8:2) or directly analysed by GC—MS after silylation (with 10 μl of BSTFA and 10 μl of pyridine for 10 min at 70°C). Chlorohydrins [trimethylsilyl(TMS) ether derivatives] were also obtained essentially as described before [15], i.e. by treatment of methyl epoxyeicosatrienoates with pyridine (20 μl) and TMCS (10 μl) for 1 h at 65°C. BSTFA (20 μl) was then added, and after 30 min at 65°C the solvents were evaporated to dryness and the residue was dissolved in hexane.

The MS analyses were performed on a Finnigan 4000 quadrupole mass spectrometer equipped with an Incos data system. An open capillary column of fused silica (20 m SE-54 CB, Arrhenius Laboratory, Stockholm University) was operated isothermally at 255°C. Splitless injections were carried out using a "falling needle" device [18]. The temperature of the ion source was set at 300°C, the electron energy was 70 eV and the emission current was 0.2 A. *C* Values were estimated from the retention times of a series of straight-chain saturated fatty acid methyl esters: methyl eicosanoate was assigned a *C* value of 20.0, methyl docosanoate a *C* value of 22.0, etc.

## RESULTS AND DISCUSSION

Methyl epoxyeicosatrienoates were converted to chlorohydrin adducts in similar yields (60—70%) either by treatment with TMCS in pyridine or by treatment with hydrochloric acid in pyridine as judged from GC after silylation. The GC—MS analysis showed that both methods also gave the same products. A slightly higher yield was obtained by treatment with hydrochloric acid in pyridine by reacting first at 65°C for 1 h and then overnight at room temperature. It is noteworthy that a solution of methoxyamine hydrochloride in pyridine, which is used to prepare methoxime derivatives of ketones, also converts epoxides to chlorohydrin adducts [11].

The mass spectra of the TMS ether derivatives of the chlorohydrin adducts of the four methyl epoxyeicosatrienoates showed a characteristic fragmenta-

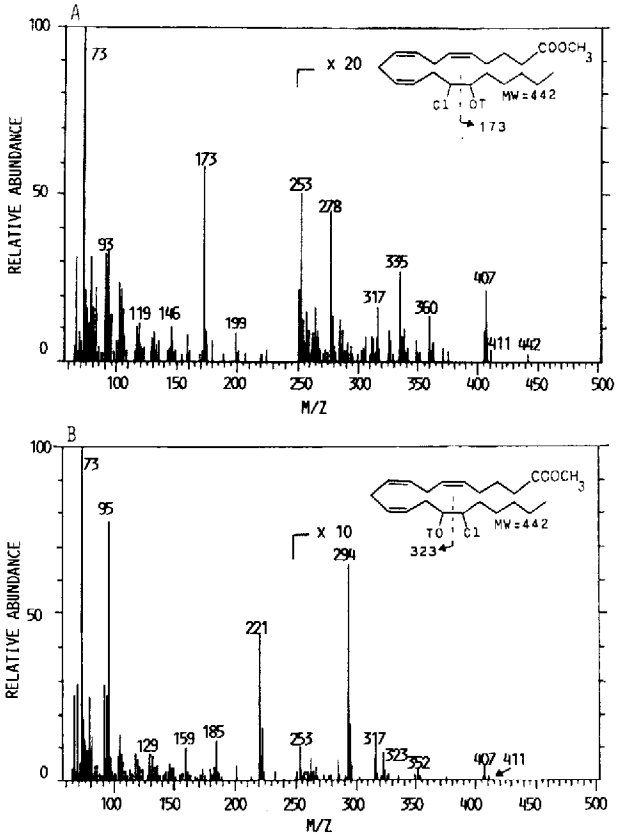


Fig. 1. Mass spectra of chlorohydrin adducts of 14(15)-epoxy-20:3 (TMS ether methyl ester derivatives). (A) 14-Chloro-15-hydroxy-20:3. (B) 14-Hydroxy-15-chloro-20:3. The insets show formation of important fragments. T stands for trimethylsilyl. The *C* values were 23.3 (A) and 23.1 (B).

tion pattern with signals at P and P + 2 for chlorine-containing fragments caused by the natural abundance of $^{35}Cl$ (76%) and $^{37}Cl$ (24%). The molecular ion (*m/z* 442) was weak as well as the signals at *m/z* 427 ($M^+ - 15$) and *m/z* 352 ($M^+ - 90$). Stronger signals were noted at *m/z* 411 ($M^+ - 31$), 407 ($M^+ -35$, loss of chlorine), 317 [$M^+ - (90 + 35)$], and *m/z* 73 was the base peak. The *C* values were 23.1 except for 5-hydroxy-6-chloro-20:3 and 14-hydroxy-15-chloro-20:3, which had a *C* value of 23.3. The mass spectra of the chlorohydrin adducts of 14(15)-epoxy-20:3, 11(12)-epoxy-20:3 and 8(9)-epoxy-20:3 are shown in Figs. 1 and 2 and will only be discussed briefly.

Methyl 14-hydroxy-15-chloro-20:3 and methyl 14-chloro-15-hydroxy-20:3 were separated by TLC ($R_F$ 0.44 and 0.5, respectively). The GC—MS analysis of the TMS ether derivative showed that the position of the TMS ether could be deduced from fragments formed by α-cleavage as shown by the insets in Fig. 1A and B (*m/z* 173 and 323, respectively). The mass spectrum of 14-hydroxy-15-chloro-20:3 (Fig. 1B) showed a strong signal at *m/z* 294, which most likely results from a rearrangement reaction, i.e. transfer of $Me_3Si$ to the carbonyl group, leading to formation of $R_m^+(Me_3Si)$, where $R_m$ denotes the side-chain formed by α-cleavage and contains the methyl ester. Corresponding

rearrangement ions in the mass spectra of 11-hydroxy-12-chloro-20:3 and 8-hydroxy-9-chloro-20:3 would be expected to give rise to signals at *m/z* 254 and *m/z* 214 (see ref. 14). The mass spectrum of 14-chloro-15-hydroxy-20:3 showed signals at *m/z* 335 [$M^+$ — (71 + 36), loss of HCl and $C_{16}$–$C_{20}$], 278 and 253. The fragments *m/z* 278 and 253 were possibly formed by rearrangement.

The chlorohydrin adducts of methyl 11(12)-epoxy-20:3 could not be resolved by TLC ($R_F$ 0.47) or by capillary GC of the TMS ether derivative under the present conditions, and this was also the case for the chlorohydrin adducts of methyl 8(9)-epoxy-20:3 ($R_F$ 0.40). The composite mass spectrum of the positional isomers of chlorohydrin adducts, which was obtained from methyl 11(12)-epoxy-20:3, is shown in Fig. 2A. Signals were noted, inter alia, at *m/z* 331 ($M^+$ — 111, loss of $C_{13}$—$C_{20}$), 295 [$M^+$ — (111 + 36), presumably loss of HCl from *m/z* 331], 254 (rearrangement as discussed above), *m/z* 261 ($M^+$ — 181, presumably loss of $C_1$—$C_{10}$) and 263, 225 (261 — 36, loss of HCl) and 205 (possibly 295 — 90). The composite mass spectrum of the positional isomers of chlorohydrin adducts derived from methyl 8(9)-epoxy-20:3 is shown in Fig. 2B. Except for the signals common to all the

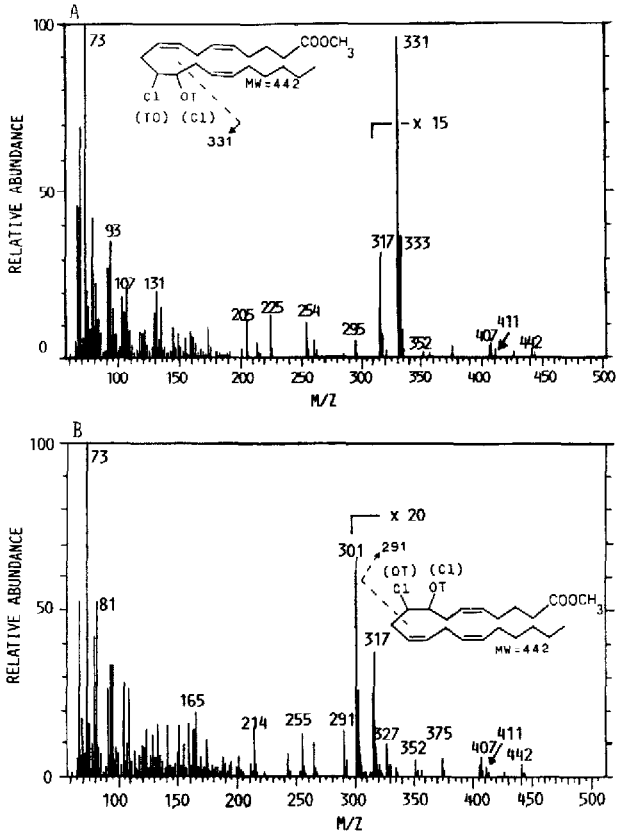


Fig. 2. Mass spectra of the chlorohydrin adducts of methyl 11(12)-epoxy-20:3 (A) and methyl 8(9)-epoxy-20:3 (B). The positional isomers of these chlorohydrins could not be resolved as indicated by the presumptive pairs of isomers of both insets. T stands for trimethylsilyl. The *C* values were 23.1.

chlorohydrin adducts, signals were noted at *m/z* 375 (possibly loss of methanol from *m/z* 407), 301 ($M^+ - 141$, loss of $C_1$–$C_7$), 291 (see inset in Fig. 2B), 255 (possibly loss of HCl from *m/z* 291) and at *m/z* 214 (rearrangement as discussed above).

The fourth epoxide, 5(6)-epoxy-20:3, has been reported to cause release of hormones in vitro [7—9]. Most remarkable is the effect of low concentrations (50 n*M*) of this epoxide on the release of luteinizing hormone from rat pituitary cells, whereas its hydrolysis product, 5,6-dihydroxy-20:3, is considerably less active [7]. 5(6)-Epoxy-20:3 can also be metabolized by fatty acid cyclooxygenase to a series of prostanoids, e.g. 5(6)-epoxy-$PGE_1$, 5-hydroxy-$PGI_{1\alpha}$ and 5-hydroxy-$PGI_{1\beta}$ (PG = prostaglandin) [10—12]. Like many other epoxides, 5(6)-epoxy-20:3 can be hydrolysed to a vicinal diol by epoxide hydrolases [4, 13]. 5(6)-Epoxy-20:3 is also chemically unstable and may be hydrolysed during the isolation procedures [10]. For these and possibly other reasons, the tissue level of this epoxide has not been determined. It therefore appeared to be of interest to evaluate the use of octadeuterated 5(6)-epoxy-20:3 as an internal standard for mass fragmentography by taking advantage of a method for trapping of epoxides by formation of chlorohydrin adducts.

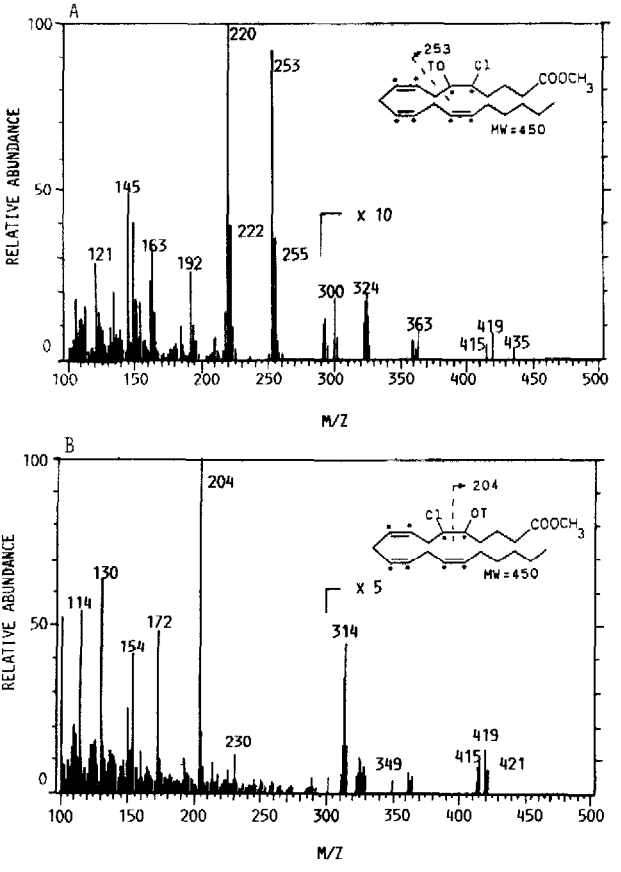


Fig. 3. Mass spectra of the chlorohydrin adducts of [$^2H_8$]5(6)-epoxy-20:3 (TMS ether methyl ester derivatives). (A) 5-Chloro-6-hydroxy-20:3. (B) 5-Hydroxy-6-chloro-20:3. The insets show formation of some important fragments. T stands for trimethylsilyl. $^2H$ is marked by an asterisk in the insets. The *C* values were 21.1 (A) and 21.3 (B).

The chlorohydrin adducts of methyl [5,6,8,9,11,12,14,15-$^2H_8$] 5(6)-epoxy-20:3 were separated by GC and their mass spectra are shown in Fig. 3. The mass spectra of the protium form of these compounds have been published elsewhere [11]. The mass spectrum of the TMS ether methyl ester derivative of [$^2H_8$] 5-chloro-6-hydroxy-20:3 showed rather weak signals at *m/z* 435 ($M^+$ — 15), 419 ($M^+$ — 31), 415 ($M^+$ — 35, loss of chlorine), 363, 324, 300 (cleavage between $C_5$ and $C_6$) and 293. The latter signal makes the fragment pair $^1H/^2H$ at *m/z* 293/300 unsuitable for mass fragmentography. In the lower mass range strong signals were noted at *m/z* 253 (conform inset in Fig. 3A) and 255, 220 (253 — 33, presumably loss of $^2HOCH_3$) and 222. The corresponding mass spectrum of [$^2H_8$] 5-hydroxy-6-chloro-20:3 showed signals, inter alia, at *m/z* 419 ($M^+$ — 31), 415 ($M^+$ — 35, loss of chlorine), 349, 324, 314, and 204 (inset). The fragment pairs $^1H/^2H$ at *m/z* 308/314 showed a ratio of 3.7%, and similar or higher $^1H/^2H$ ratios were obtained for most fragment pairs in the middle mass range of both chlorohydrins. The fragment pairs $^1H/^2H$ at *m/z* 411/419 ($M^+$ — 31) and 407/415 ($M^+$ — 35) showed a ratio of less than 1.2%, gave linear standard curves and thus appeared to be of value for mass fragmentography.

The present results demonstrate that epoxyeicosatrienoic acids can be trapped as chlorohydrin adducts simply by treatment with hydrochloric acid in pyridine. After conversion to TMS ether derivatives the chlorohydrin adducts give rise to mass spectra that are more informative than those of the parent compounds and have proved to be of value for identification of 5(6)-epoxy-$PGE_1$ and 5(6)-epoxy-heptadecadienoic acid [11, 12]. Chlorohydrins may also be obtained by treatment with TMCS in pyridine, but by this method TMS ether derivatives are formed that are unsuitable for subsequent purification by HPLC or by TLC. Recently, the use of [$^2H_3$] methyl ester derivatives of epoxyeicosatrienoic acids as internal standards for mass fragmentographic analysis of epoxyeicosatrienoic acids was reported [19]. By this method, the $^1H/^2H$ ratio of the internal standard was 9%. In comparison, the use of octadeuterated 5(6)-epoxy-20:3 as an internal standard for 5(6)-epoxy-20:3 with the subsequent conversion to chlorohydrin adducts gives rise to fewer ions in the upper mass range that have *m/z* values matching those from the analyte and may prove useful for the determination of this epoxide in tissues.

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REFERENCES

1 B. Samuelsson, Science, 22 (1983) 568.
2 E.H. Oliw, J.A. Lawson, A.R. Brash and J.A. Oates, J. Biol. Chem., 256 (1981) 9924.
3 E.H. Oliw and J.A. Oates, Biochim. Biophys. Acta, 666 (1981) 327.
4 E.H. Oliw, F.P. Guengerich and J.A. Oates, J. Biol. Chem., 257 (1982) 3771.

5 N. Chacos, J.R. Falck, C. Wixtrom and J. Capdevila, Biochem. Biophys. Res. Commun., 104 (1982) 916.
6 E.H. Oliw and P. Moldeus, Biochim. Biophys. Acta, 721 (1982) 135.
7 G.D. Snyder, J. Capdevila, N. Chacos, S. Manna and J.R. Falck, Proc. Nat. Acad. Sci. U.S., 80 (1983) 3504.
8 J.R. Falck, S. Manna, J. Moltz, N. Chacos and J. Capdevila, Biochem. Biophys. Res. Commun., 114 (1983) 743.
9 J. Capdevila, N. Chacos, J.R. Falck, S. Manna, A. Negro-Vilar and S.R. Ojeda, Endocrinology, 113 (1983) 421.
10 E.H. Oliw, J. Biol. Chem., 259 (1984) 2716.
11 E.H. Oliw, Biochim. Biophys. Acta, 793 (1984) 408.
12 E.H. Oliw, FEBS Lett., 172 (1984) 279.
13 N. Chacos, J. Capdevila, J.R. Falck, S. Manna, C. Martin-Wixtrom, S.S. Gill, B.D. Hammock and R.W. Estabrook, Arch. Biochem. Biophys., 223 (1983) 639.
14 E.H. Oliw, J. Chromatogr., 275 (1983) 245.
15 D.J. Harvey, D.B. Johnson and M.G. Horning, Anal. Lett. 5 (1972) 745.
16 E.J. Corey, H. Niwa and J.R. Falck, J. Amer. Chem. Soc., 101 (1979) 1586.
17 E.J. Corey, J.O. Albright, A.E. Barton and S. Hashimoto, J. Amer. Chem. Soc., 102 (1980) 1435.
18 P.M.J. van den Berg and T.P.H. Cox, Chromatographia, 5 (1972) 301.
19 J. Capdevila, B. Pramanik, J.L. Napoli, S. Manna and J.R. Falck, Arch. Biochem. Biophys., 231 (1984) 511.