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Evaluation of different injection techniques in the gas chromatographic determination of thermolabile trace impurities in a drug substance

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Abstract

A gas chromatographic method for the determination of an epoxide in a drug substance was validated. Hot splitless injection of the sample extract was identified as a critical step with unsatisfactory repeatability. Therefore, the gas chromatographic procedure was re-evaluated using an instrument equipped with an on-column inlet and a split–splitless inlet with a programmable column head pressure. Further, the re-evaluated method also included the determination of the corresponding chlorohydrin that might occur as a reaction product of the epoxide. Three different sets of parameters were chosen: splitless injection using the same parameters as were used in the validation experiments, splitless injection with a high initial column head pressure and cool on-column injection. Generally, the precision of repeated injections of standard solutions and of the analysis of spiked sample extracts was improved using the newer instrument. On-column injection gave the best results in terms of both precision and absolute peak areas. The limits of detection and quantification for the overall method were 0.09 and 0.29 μg/g, respectively, for the epoxide and 0.09 and 0.31 μg/g, respectively, for the chlorohydrin. On hot splitless injection the chlorohydrin formed the epoxide, and also losses of the epoxide were observed. Owing to a shorter residence time in the hot injector block, a high initial column head pressure could successfully reduce the degradation of the analytes. However, the precision was not improved because of occasional leakage of the septum immediately after injection, which resulted in uncontrolled losses and discrimination.

1. Introduction

Epoxides are potentially toxic, mutagenic and carcinogenic agents. The presence of ethylene oxide residuals in food stuffs and pharmaceuticals is regulated by the authorities in many countries owing to its known carcinogenic potential in humans. The limit is typically 1 ppm. In drug substances any epoxide that might be present must be regarded as a toxic impurity although it might be less reactive than ethylene oxide. The concentration should be kept as low as possible, and a limit will usually be included in the specification of the particular drug substance. Individual identification and specific test methods are then required. The analytical test method must be validated especially for selectivity and limits of detection and quantification.

The epoxide H 137/89 [*p*-cyano(epoxypropoxy)benzene], is involved in the synthesis of Almokalant, an antiarrhythmic drug in the clinical phase of investigation. A validated analytical method was needed for its determination in the region of 1 μg/g, which was close to the ex-

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pected limit of quantification. Further, there was interest in determining, with the same method, the corresponding chlorohydrin H 240/18 [*p*-cyano(3 - chloro - 2 - hydroxypropoxy)benzene], which is a possible reaction product of H 137/89. Chemical structures are presented in Fig. 1.

The limits of detection and quantification in trace analysis with chromatographic methods depend on the selectivity and precision of the method and on the performance of the chromatographic system. The method to be applied for the determination of the epoxide and chlorohydrin was capillary gas chromatography using a non-polar methylsilicone as the stationary phase. Epoxide and chlorohydrin were extracted from the main compound by liquid–liquid extraction with dichloromethane. Almokalant has a pK_a value of 7.8 and can be kept in the aqueous phase by adding an excess of phosphoric acid. During validation of the method for the determination of epoxide, splitless injection turned out to be a critical step for the method precision, and thermodegradation was considered as a reason. The method was therefore re-evaluated using a second gas chromatographic system equipped with an on-column inlet and a split–splitless inlet with programmable column head pressure. Cool on-column injection is regarded as the "smoothest" injection technique as the sample enters the column without previous volatilization and at low initial temperature. Hence it is the method of choice for thermolabile samples [1,2], provided that the sample is sufficiently clean, to avoid interfering matrix effects. In splitless injection pressure programming can reduce the residence time of the vaporized sample in the hot injection port and hence thermodegradation can be minimized [3]. In this work, different gas chromatographic methods were tested and optimized. In order to find the most suitable method for the determination of both substances, parts of the validation experiments were repeated and the results from different gas chromatographic methods were compared.

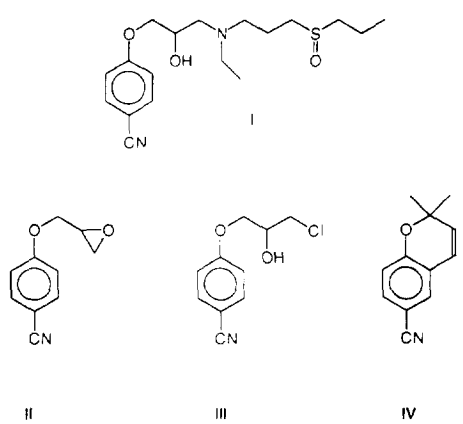

Fig. 1. Structural formulae of Almokalant (I), epoxide H 137/89 (II), chlorohydrin H 240/18 (III) and the internal standard (IV).

2. Experimental

A solution of about 4 μg/ml H 254/89 (6-cyano-2,2-dimethyl-2*H*-1-benzopyran, Fig. 1) in dichloromethane was prepared and used as an internal standard solution. Working standard solutions of epoxide H 137/89 and chlorohydrin H 240/18 were prepared from stock standard solutions by dilution with the internal standard solution. The sample (0.95–1.05 g of Almokalant) was weighed into a vial equipped with a Teflon-lined screw-cap and spiked with an appropriate amount of the standard solutions. A 3.00-ml volume of internal standard solution and 3.00 ml of 1 *M* phosphoric acid were added and the vial was shaken vigorously for 5 min and centrifuged for 5 min at about 2500 rpm (ca. 800 *g*). The upper phase was removed and 3.00 ml of 0.1 *M* phosphoric acid were added. The vial was shaken again for 5 min. After centrifugation, the upper phase was removed and the organic phase was transferred into another vial where it was dried over about 5 mg of anhydrous sodium sulfate. Standard solutions and sample extracts were injected into the gas chromatograph.

The validation experiments were performed using a Hewlett-Packard HP 5790 gas chromatograph equipped with a capillary column split–splitless injector. The gas chromatographic parameters were as follows: column, 25 m × 0.32

mm I.D. fused-silica capillary column coated with 0.52-μm cross-linked methylsilicone (Hewlett Packard Ultra 1); temperatures, injector 200°C, detector 280°C, oven initial temperature 40°C (held for 2 min), increased at 12°C/min to 270°C (held for 5 min); carrier gas, helium at 2.0 ml/min (0.71 bar); splitting ratio, 1:15; detector auxiliary gas, nitrogen at 35 ml/min; and detector gases, hydrogen at 35 ml/min and air at 240 ml/min.

The second instrument was a Hewlett-Packard HP 5890 Series II gas chromatograph equipped with a flame ionization detector, a capillary column split–splitless injector and an on-column injector. The same column was used but it was coupled to a 5 m × 0.32 mm I.D. fused-silica capillary retention gap. Detector gases and detector temperatures were the same as described above. The initial carrier gas (helium) flow-rate was 1.8 ml/min and the column head pressure was 0.69 bar. The temperature programmes were similar in all methods used.

The sample was injected manually, either splitless or on-column, with a 10-μl syringe with a removable fused-silica needle. For splitless injections, deactivated, straight glass liners, 79 mm × 4 mm I.D., with a small plug of deactivated glass-wool were used. The glass-wool was positioned about half way down the liner. The syringe needle length was 50 mm and it placed the sample at or slightly above the glass-wool plug. Injection of 2-μl samples, needle volume included, was performed using the hot needle technique. The plunger was pushed down rapidly and the needle was withdrawn from the inlet immediately after injection. This is regarded as the most reproducible manual injection technique.

3. Results and discussion

3.1. Validation experiments

The described method was validated with respect to chromatographic selectivity, linearity, recovery, precision and limits of detection and quantification. The chlorohydrin was not included in the validation experiments.

A chromatogram of a sample spiked with 10 μg/g of epoxide run on the HP 5790 gas chromatograph is shown in Fig. 2. An unspiked blank sample was prepared in the same manner. The dichloromethane extract was reduced to *ca.* 1 ml by blowing with nitrogen and injected into the gas chromatograph. The blank was free of detectable concentrations of the epoxide.

The recovery of the epoxide in spiked samples of Almokalant was studied at three concentration levels between 1 and 10 μg/g. Samples of 1.00 ± 0.05 g were weighed and spiked with standard solution. The extraction was performed according to the method described above and the concentration of the epoxide in the organic phase was calculated. An extraction yield of

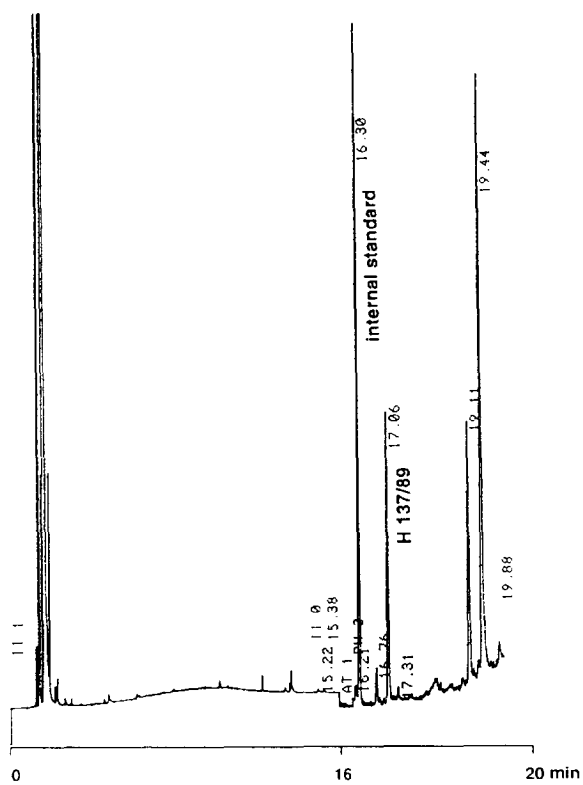

Fig. 2. Chromatogram of a sample of Almokalant spiked with 10 μg/g of epoxide H 137/89 and run on the HP 5790 gas chromatograph.

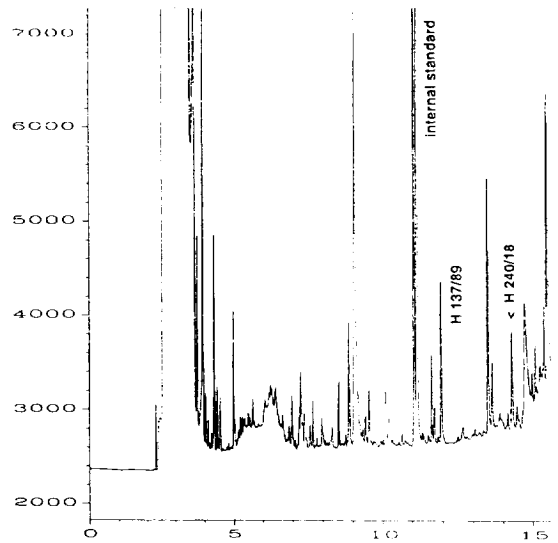

Fig. 3. Chromatogram of a sample of Almokalant spiked with 1 μg/g of epoxide H 137/89 and chlorohydrin H 240/18 and injected on-column on to the HP 5890 Series II gas chromatograph. Time scale in min. Values on the y-axis in pA.

88.5% was obtained as the percentage of added epoxide that could be recovered in the organic phase.

The repeatability of the method was determined at a concentration close to the limit of quantification. Five samples of Almokalant were spiked with epoxide stock standard solution to give a final concentration of about 1 μg/g. These samples were also used as the lowest concentration level for the determination of the extraction yield. The peak-area ratios of epoxide and internal standard were calculated for the five samples. The relative standard deviation of the peak area ratio of epoxide and internal standard was 9.2%.

The method limit of detection (MLD) was defined as the concentration (μg/g) giving a peak-area ratio of the epoxide to the internal standard equal to three times the standard deviation of the peak-area ratio of a concentration slightly above the expected limit of quantification. The limit of quantification was defined as the concentration (in μg/g) giving a peak-area ratio of the epoxide to the internal standard equal to ten times the standard deviation of the peak area ratio of a concentration slightly above the expected limit of quantification (according to Taylor [4]). Based on the data obtained in the precision experiments, the method limit of detection was 0.3 μg/g and the limit of quantification was 0.9 μg/g.

The extraction step was studied in more detail in a number of additional experiments. Three samples were spiked with the same amount of epoxide (*ca.* 10 μg/g) and extracted in different ways. One sample was shaken manually for 5 min and the other two were shaken with a shaking apparatus at 350 rpm for 5 min and for 30 min, respectively. The sample shaken for 30 min showed a slightly lower recovery (80.2%) than the samples shaken for 5 min. This may be a result of hydrolysis of the epoxide. Manual shaking or use of a shaking apparatus had no effect on the extraction yield. Further, the extractability of the internal standard from dichloromethane to phosphoric acid was tested in the presence of the sample matrix. About 1 g of Almokalant was weighed and 3 ml of the internal standard solution were added. After addition of 3 ml of 1 M H_3PO_4, the vial was shaken vigorously for 5 min and the phases were separated by centrifugation. The aqueous phase was transferred to a new vial and shaken with 3 ml of dichloromethane without internal standard for 5 min. The dichloromethane phase was injected into the gas chromatograph. No internal standard was detectable. Hence the internal standard was not likely to be extracted into the aqueous phase during sample work-up.

3.2. Re-evaluation

Although the validated limit of quantification was sufficient, the precision of the method was not satisfactory. A number of experiments were carried out in order to find the critical parameters. However, removal of the glass-wool from the glass liner, changing the glass liner, variation of the injector temperature (180 and 220°C), variation of the splitless time and injection of 1 μl instead of 2 μl did not improve the precision of repeated injections of standard solutions of either epoxide or chlorohydrin. It was obvious from the above experiments that the chloro-

hydrin was subject to thermodegradation and formed epoxide by release of HCl. The peak shape indicated that no degradation occurred on the column so that the sample introduction system seemed to be the place of degradation, for example, as a result of active sites within the injection system or simply by heat [2,5]. It was also observed that the absolute peak areas in some instances varied by more than 10% relative standard deviation. Therefore, the gas chromatographic process was re-evaluated using a newer instrument (HP 5890 Series II gas chromatograph) equipped with both a capillary column split–splitless injector and an on-column injector, and electronic pressure programming of the column head pressure. Additionally, determination of the chlorohydrin H 240/18 in Almokalant was included in the method.

Three different methods were tested:

(I) (splitless). Similar parameters as described above for the HP 5790 instrument, splitless injection.

(II) (electronic pressure programming, EPP). A high inlet pressure during injection (2.2 bar, 30 s) in order to obtain a high inlet-flow rate and thus reduce possible thermal decomposition of the sample during splitless injection.

(III) (cool on-column injection). The instrument parameters were similar to those in methods I and II.

The use of a retention gap in the re-evaluation experiments did not influence the peak shape; it was merely used in order to protect the column from contamination by the sample extracts injected on-column. In the on-column experiments epoxide and chlorohydrin were determined in the same run whereas in the splitless experiments epoxide and chlorohydrin were run separately. The precision of the injection was determined from repeated injections of standard solutions containing 5 μg/ml of epoxide or chlorohydrin. The precision of the overall method was tested as described above with repeated samples spiked with epoxide or chlorohydrin at the 1 μg/g level. However, in the re-evaluation experiments no epoxide- and chlorohydrin-free Almokalant batch was available. The available batch contained 0.54 μg/g of epoxide and 0.69 μg/g of chlorohydrin, determined with the on-column method. The true concentration levels of the spiked samples are therefore about 1.5 μg/g of epoxide and 1.7 μg/g of chlorohydrin. For the same reason, the extraction yield of the chlorohydrin could not be determined and was suggested to be 100%. As the same blank was used for all re-evaluation experiments, this did not influence the final results. The results are summarized in Table 1 and compared with the results obtained from the HP 5790 gas chromatograph. A chromatogram of a sample of Almokalant spiked with 1 μg/g of epoxide and chlorohydrin and injected on column is shown in Fig. 3.

Using similar parameters on the HP 5790 and 5890 instruments, the relative standard deviation of repeated splitless injections of the epoxide was improved considerably on the HP 5890 and the relative standard deviation of the overall method could be lowered from 9.2 to 2.6%. According to the manufacturer, the pneumatic systems are identical for the two instrument models. However, an important difference in injector design is the insulation between injector block and column oven. In the older instrument a temperature gradient could develop along the hot injector owing to insufficient insulation from the cooler oven. Condensation of higher boiling components in the lower part of the injector could result in a lower precision in sample transfer. In the HP 5890 instrument the injector insulation was improved and an aluminium cap was installed which additionally prevents heat exchange between the two compartments of the gas chromatograph (information from Hewlett-Packard, Stockholm, Sweden).

Injection of the chlorohydrin standard solutions showed that it degraded during injection and formed epoxide. As can be seen in Table 2, on normal splitless injection (method I) 26% of the injected chlorohydrin formed epoxide. A higher inlet pressure during injection, shortening the residence time of the vaporized sample in the injection block, decreased the formation of epoxide to 9%. With an increase in the inlet temperature from 200 to 230°C, epoxide formation increased to 37%. A chromatogram of this sample is shown in Fig. 4. In contrast, no epoxide formation from a pure chlorohydrin

Table 1
Comparison of different methods for the determination of the epoxide H 137/89 and the chlorohydrin H 240/18 in Almokalant

GC model (method)	Epoxide				Chlorohydrin			
	R.S.D.$_{inj}$ (%)	R.S.D.$_{met}$ (%)	LOD (μg/g)	LOQ (μg/g)	R.S.D.$_{inj}$ (%)	R.S.D.$_{met}$ (%)	LOD (μg/g)	LOQ (μg/g)
HP 5790 (splitless)	3.7 ($n = 7$)	9.2 ($n = 5$)	0.3	0.9	–	–	–	–
HP 5890 (I, splitless)	0.39 ($n = 3$)	2.6 ($n = 5$)	0.13	0.42	6.7 ($n = 3$)	3.1 ($n = 5$)	0.17	0.56
HP 5890 (II, EPP)	0.32 ($n = 3$)	4.3 ($n = 5$)	0.22	0.72	3.8 ($n = 3$)	6.9 ($n = 5$)	0.35	1.2
HP 5890 (III, on-column)	0.19 ($n = 3$)	1.8 ($n = 5$)	0.09	0.29	0.64 ($n = 3$)	2.1 ($n = 5$)	0.09	0.31

R.S.D.$_{inj}$ = Relative standard deviation of the peak-area ratio of the epoxide (chlorohydrin) to the internal standard for repeated injection of a standard solution (5 μg/ml); R.S.D.$_{met}$ = relative standard deviation of the peak-area ratio of epoxide (chlorohydrin) to the internal standard in repeated samples spiked with 1 μg/g of epoxide or chlorohydrin; LOD = limit of detection; LOQ = limit of quantification.

standard was observed during on-column injection. The dependence on injector temperature and residence time in the injector block of the chlorohydrin peak area can also be seen in Table 3, where absolute peak areas are compared.

Although the thermolability of the chlorohydrin is more obvious, a slight thermolability of the epoxide is also indicated from the absolute peak areas shown in Table 3. Compared with the on-column method the peak areas of the epoxide are decreased by between 6.5 and 17%, depending on the method, whereas the differences in internal standard peak areas between on-column and splitless methods are considerably lower.

Table 2
Formation of epoxide from chlorohydrin standard with different methods on the HP 5890 gas chromatograph

Method	Epoxide formed (%)[a]
I (splitless)	26 (injector temp. 200°C)
	37 (injector temp. 230°C)
II (EPP)	9
III (on-column)	0

[a] Percentage of chlorohydrin injected that has been converted into epoxide.

The losses of chlorohydrin during splitless injection cannot be explained only by epoxide formation since the amount epoxide recovered is lower than the corresponding loss of chlorohydrin compared with on-column injection. A possible

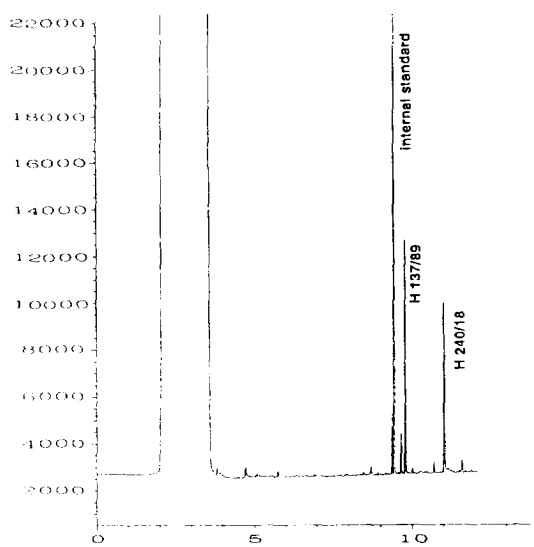

Fig. 4. Chromatogram of a standard solution of chlorohydrin H 240/18 injected on to the HP 5890 instrument at an injector temperature of 230 instead of 200°C used in method I. The epoxide H 137/89 was formed from the injected chlorohydrin in the hot injector block and corresponds 37% of the chlorohydrin. Scales as in Fig. 3.

Table 3
Absolute areas ± relative standard deviations of internal standard, epoxide and chlorohydrin peaks obtained from repeated injections of standard solutions using different methods

Method	Epoxide		Chlorohydrin	
	Area internal standard	Area epoxide	Area internal standard	Area chlorohydrin
I (splitless)	31 519 ± 4.1% ($n = 3$)	32 010 ± 4.7% ($n = 3$)	30 329 ± 4.6% ($n = 3$)	11 637 ± 5.5% ($n = 3$)
II (EPP)	33 127 ± 3.5% ($n = 3$)	34 922 ± 3.7% ($n = 3$)	32 826 ± 3.5% ($n = 3$)	23 638 ± 1.8% ($n = 3$)
III (on-column)	32 310 ± 0.61% ($n = 3$)	37 333 ± 0.75% ($n = 3$)	32 310 ± 0.61% ($n = 3$)	28 882 ± 0.94% ($n = 3$)

reason is the formation of degradation products other than epoxide, adsorption in the injection system, which might affect both epoxide and chlorohydrin, or any kind of discrimination. If discrimination occurs it will affect the relative response of the chlorohydrin more than that of the epoxide, as the latter has a retention time closer to that of the internal standard.

The use of a high initial column head pressure was very effective in decreasing chlorohydrin losses during splitless injection, and absolute peak areas of the chlorohydrin peak could be more than doubled by using this technique. The loss relative to on-column injection was 18%, compared with 60% with normal initial pressure. However, problems occurred owing to septum leaks that occurred occasionally and resulted in severe discrimination. Changing the septum did not immediately improve the injection and the system seemed to be very sensitive to factors such as the type of septum used, the number of injections made through the same septum, the tightness of the septum retainer nut and the speed at which the syringe needle was withdrawn from the injection port. Samples for which a septum leak was observed were re-injected. An automatic injector might improve this type of problem.

On-column injection is known to improve precision and accuracy and to eliminate problems resulting from discrimination [2]. Comparing the different methods, on-column injection gave best results in terms of both precision and absolute peak areas. Hence, the method limits of detection and the limits of quantification could be lowered. When chlorohydrin is present in the sample it will form epoxide on splitless injection and therefore on-column injection is required in order to produce reliable results.

4. Conclusions

Generally, the precision of the injection and of the overall method was improved considerably using the HP 5890 instrument. The reason might be improved insulation between the injector and the gas chromatograph oven. High initial inlet pressure during injection, effective in decreasing thermodegradation of the chlorohydrin, did not improve the precision. The increased pressure led to occasional leakage of the septum immediately after injection, which resulted in uncontrolled losses and discrimination.

The results indicate that the chlorohydrin and, to a minor extent, the epoxide suffer from thermal degradation and/or adsorption in the hot injector block during splitless injection, which results in poor precision. Consequently, the best results regarding precision and limits of detection and quantification were obtained by using cool on-column injection. When chlorohydrin is present in the sample it will form epoxide on splitless injection and hence on-column injection becomes the method of choice.

References

[1] H.-M. Müller and H.-J. Stan, *J. High Resolut. Chromatogr.*, 13 (1990) 759.
[2] K. Grob, *Classical Split and Splitless Injection in capillary GC*, Hüthig, Heidelberg, 2nd ed., 1988, pp. 176–188.
[3] P.L. Wylie, K.J. Klein, M.Q. Thompson and B.W. Hermann, *J. High Resolut. Chromatogr.*, 15 (1992) 763.
[4] J.K. Taylor, *Quality Assurance of Chemical Measurements*, Lewis, Chelsea, MI, 1987, p. 79.
[5] H. Husmann, G. Schomburg, K.-D. Müller, H.P. Nalik and G. von Recklinghausen, *J. High Resolut. Chromatogr.*, 13 (1990) 780.