Simultaneous Quantitative Determination of Amiloride Hydrochloride and Hydrochlorothiazide in Tablets by High-Performance Liquid Chromatography

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Key Words

Column liquid chromatography
Amiloride hydrochloride
Hydrochlorothiazide
Quantitative determination

Summary

A procedure for the simultaneous quantitative determination of amiloride hydrochloride and hydrochlorothiazide by high-performance liquid chromatography is proposed. A reversed-phase LiChrosorb C8 stationary phase is used. The eluent consisted of an acetonitrile/0.1M phosphate buffer pH 3 (15:85) mixture, containing 50mM propylamine hydrochloride. In this system amiloride hydrochloride, a basic drug, eluted with an acceptable asymmetry factor (Asf = 2.1). A simple extraction procedure with methanol is used. Relative standard deviations of 0.87% and 1.6% were obtained for amiloride hydrochloride and hydrochlorothiazide respectively. Chlorothiazide, a thiazide diuretic, is a suitable internal standard. Furthermore the method is also specific for other thiazide diuretics, potassium-sparing diuretics and loop diuretics and for the respective hydrolysis products of both drugs. Analysis time is reduced to a minimum; the chromatographic separation is complete within 6 minutes.

Introduction

Hydrochlorothiazide (HCT – a thiazide diuretic) and amiloride hydrochloride (AMI – a potassium-sparing diuretic) are often administered together for the treatment of hypertension. Until now only a spectrophotometric method has been described for the simultaneous determination of the two compounds [1]. This method lacks specificity, the hydrolysis product of HCT, 4-amino-6-chlorobenzene-1, 3-disulphonamide also absorbs UV at 270nm, the wavelength used in spectrophotometric determinations of HCT. AMI has been determined by a photometric method [2, 3] and by a thin-layer chromatographic method with fluorescence measurement [4]. Only recently a liquid chromatographic method was described for pharmacokinetic studies [5]. No mention was made of the hydrolysis product of AMI. Several papers describe the determination of HCT alone using different techniques [6–11]. This paper describes the development of a specific liquid chromatographic method for the simultaneous determination of the two compounds.

Experimental

1 Chemicals and Reagents

All reagents were of analytical grade quality. Acetonitrile was of HPLC grade quality. Amiloride hydrochloride (AMI) and hydrochlorothiazide (HCT) were assayed in Moduretic® tablets (Merck, Sharp & Dohme, Haarlem, The Netherlands) and in an experimental formulation. The label claim was respectively 5mg and 50mg per tablet.

2 HPLC Equipment and Column

An SP 8770 isocratic pump (Spectra Physics, Darmstadt, FRG) was used equipped with an HP 1040A photo diode array detector (Hewlett Packard, Palo Alto, CA, USA), an HP 85 computer, an HP 82901 flexible disc drive and an HP 3390A integrator. The HP 1040A is a high-speed spectrophotometric detector; it takes less than 1sec to measure a UV spectrum and enabled us to take UV spectra of the compounds as they eluted from the column. The spectra can be stored on a disc so that after the chromatographic run they can be evaluated. Spectra stored on a disc can be plotted over each other to see if they coincide.

The temperature of the column is maintained constant with a water bath. A LiChrosorb RP C 8 and C 18 column (5μm) of the same dimensions were used. The column type and the chromatographic conditions for quantitative determination are listed in Table I. The asymmetry factor was measured at 10% peak height, a perpendicular was drawn from the peak maximum to the baseline. The rear portion of the peak was divided by the front portion at 10% peak height.

3 Preparation of the Mobile Phase

The ratios of organic solvent : water mixtures refer to volume ratios. The acetonitrile : buffer eluents containing an amine additive were prepared as follows: the amine additive and phosphoric acid were dissolved in water, the pH of the solution was adjusted to the desired value with 1M sodium

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Table I HPLC conditions for the quantitative determination of amiloride hydrochloride and hydrochlorothiazide.

Column	LiChrosorb RP C8, 5μm, 150 X 4.6mm
Eluent	acetonitrile/0.1M phosphate buffer pH 3 (15:85), containing 50mM propylamine hydrochloride; eluent is filtered through 5μm filter and degassed with helium
Column temperature	25°C
Flow rate	1 ml min^{-1}
Sample loop	10μl
Detector	U. V. detector, wavelength 286nm, sensitivity 0.1 AUFS
Recorder	chart speed 0.5 cm min^{-1}

hydroxide, then the stated volumes of acetonitrile and of the water phase were mixed. The molarity of the phosphate buffer refers to the water phase. The indicated molarity of the amine additive refers to the molarity in the total eluent.

4 Determination of Amiloride Hydrochloride and Hydrochlorothiazide

a Internal standard solution

A solution of chlorothiazide (CTZ – 0.2mg ml^{-1}) in methanol was used.

b Standard solution

Two concentrated solutions in methanol were prepared containing respectively 0.5mg AMI ml^{-1} and 2.0mg HCT ml^{-1}. These solutions were freshly prepared. A working standard solution was prepared by mixing 2.0ml concentrated AMI solution, 5.0ml concentrated HCT solution with 10.0ml internal standard solution and 3ml methanol and diluting to 100ml with acetonitrile:water (15:85).

c Sample preparation

Twenty tablets were ground.

An amount of ground tablet powder, corresponding to 5mg AMI and 50mg HCT was weighed into a 50ml volumetric flask, mixed and diluted to 50ml with methanol.

This solution was stirred for 5min and then centrifuged at 3000g for 5min. 5.0ml of the supernatant and 5.0ml internal standard solution were mixed and diluted to 50ml with acetonitrile:water (15:85).

Results and Discussion

1 Optimization of Elution of Amiloride Hydrochloride and Hydrochlorothiazide

The purpose of this paper was to develop a specific, fast and reproducible determination of both AMI and HCT in a single run. The mobile phase was therefore optimized; the influence of the pH of the eluent and the nature and concentration of an amine additive in the eluent are discussed.

In a previous paper the influence of the pH of an eluent consisting of acetonitrile:0.05M phosphate buffer (40:60) was investigated using a LiChrosorb C 18 stationary phase [12]. An acidic pH was selected for the quantitative determination of AMI (Fig. 1A). At pH 3 AMI, a basic compound, is positively charged (pKa 8.7) [13]. A reduced silanophilic interaction is observed in an acidic medium due to the reduced dissociation of the silanol groups, resulting in better peak symmetry. For obvious reasons (breakdown of the reversed-phase packing) [14, 15] a basic eluent (pH > 11) was not used; at this pH AMI should elute in non-ionised form with a better peak symmetry [15].

The addition of propylamine hydrochloride, a competing base, to the eluent was necessary to improve the peak shape of AMI. This causes AMI to elute even faster than HCT (Fig. 1B) [12, 16]. At 15mM propylamine hydrochloride AMI elutes close to the dead volume of the column. The

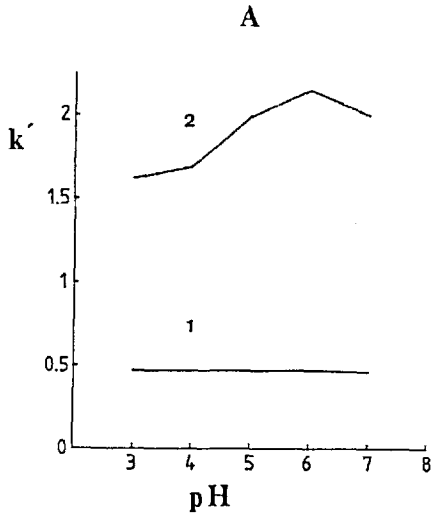


Fig. 1A

Influence of pH of eluent on retention of HCT (1) and AMI (2). Chromatographic conditions: see Table I except mobile phase (acetonitrile/0.05M phosphate buffer (40:60)) and column (reversed-phase C18 column).

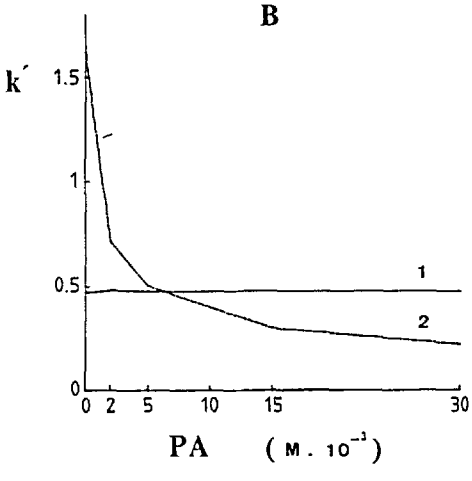


Fig. 1B

Influence of concentration of propylamine hydrochloride in eluent consisting of acetonitrile/0.05M phosphate buffer pH 3 (40:60) on retention of HCT (1) and AMI (2).

Chromatographic conditions: see Table I; except column reversed-phase C18.

acetonitrile/phosphate buffer ratio was then adjusted to elute AMI with a larger k′ value.

With a mobile phase consisting of acetonitrile:0.05M phosphate buffer, pH 3, (15:85) containing 15mM propylamine hydrochloride reasonable k′ values were obtained for AMI (k′ 1.24) and HCT (k′ 2.20). An asymmetry factor (Asf) of 3.6 for AMI in a concentration of 0.05mgml^{-1} was, however, still too high for precise and reproducible quantitation of AMI. Different conditions were examined to decrease the Asf of AMI; the effect on the Asf and on the k′ value was investigated (Table II).

An increase of the molarity of propylamine hydrochloride in the eluent has an effect on the Asf comparable to an increase of the acetonitrile content of the eluent. Indeed for the same decrease in k′ value in the two systems the same Asf was obtained. Increasing the temperature is also good for reducing the Asf but quite high temperatures are needed. The nature of the amine additive to the eluent also plays an important role. Sokolowski and Wahlund [18] found that bulky additives gave little or no improvement in the peak symmetry.

We compared the reducing effect of propylamine hydrochloride on tailing to dimethylethylamine which was one of the best amine additives [17]. Table II shows that in the presence of dimethylethylamine the k′ value decreased to a larger extent but the Asf did not. This shows that propylamine hydrochloride is more effective in reducing tailing than dimethylethylamine. We therefore selected propylamine hydrochloride as the additive.

The influence of the concentration of AMI on the peak symmetry is shown in Fig. 2. As the concentration of AMI increases, the Asf increases in the same sense with both amines. In the presence of 50mM propylamine hydrochloride, the Asf is still 3.1 for AMI. We therefore tried a reversed-phase C 8 column since the shorter alkyl chains might provide better accessibility to the surface silanol groups. As stated by Bidlingmeyer *et al.* [18] the accessibility of the silanol groups plays an important role in the peak symmetry and not the number of residual silanol groups. The results are summarized in Table III. Compared to the reversed-phase C18 column the Asf of AMI has nearly the same value for an eluent containing 15mM propylamine hydrochloride at pH3. Increasing the amine content or the acetonitrile content in the eluent has a remarkable effect on the Asf of AMI. Indeed, at 50mM propylamine hydrochloride an acceptable Asf of 2.1 was obtained. Increasing the pH has the opposite effect. At

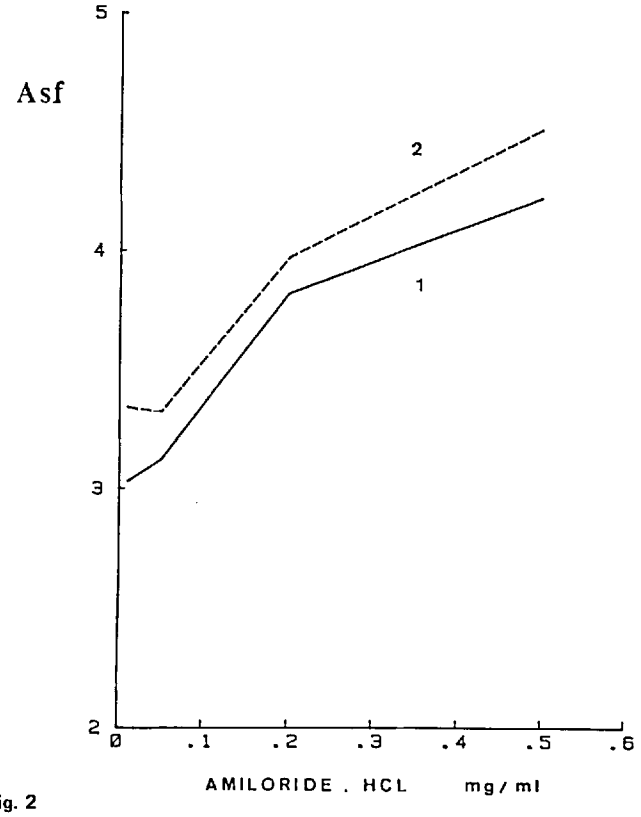


Fig. 2

Influence of concentration of AMI in sample solution on asymmetry factor. Mobile phase: acetonitrile/0.1M phosphate buffer pH 3 (15:85) containing 50mM propylamine hydrochloride (1) or 50mM dimethylethylamine (2) as amine additive.

Chromatographic conditions: see Table I; except column reversed-phase C18.

Table II Influence of different conditions on Asf of AMI on reversed-phase C18 column.

Mobile phase: acetonitrile/0.05M phosphate buffer pH 3 (15:85) containing 15mM propylamine hydrochloride; Column temperature: 25°C; Concentration of AMI: 0.05mgml^{-1}.

Conditions changed	Value	AMI	
		Asf	k′
–	–	3.6	1.24
mM propylamine hydrochloride M phosphate buffer	50 0.1	3.1	0.77
Temperature °C	35	3.3	0.97
Amine additive: dimethylethylamine: mM M phosphate buffer	50 0.1	3.3	0.74
Acetonitrile/buffer ratio	20:80	3.2	0.78

Table III Influence of different conditions on Asf of AMI on reversed-phase C8 column.

Mobile phase: acetonitrile/0.05M phosphate buffer pH 3 (15:85) containing 15mM propylamine hydrochloride; Column temperature: 25°C; Concentration of AMI: 0.05mgml^{-1}

Conditions changed	Value	AMI	
		Asf	k′
–	–	3.5	1.34
mM propylamine hydrochloride M phosphate buffer	50 0.1	2.1	1.02
mM propylamine hydrochloride M phosphate buffer pH of the phosphate buffer	50 0.1 4	3.0	1.26
mM propylamine hydrochloride M phosphate buffer pH of the phosphate buffer	50 0.1 2	2.4	0.89
Acetonitrile/buffer ratio	20:80	2.4	0.68

pH 4 the dissociation of the silanols becomes more important and results in an increase in the Asf. Decreasing the pH to 2 gives no improvement. In addition this pH is close to the minimum pH normally used for reversed-phase materials. On the basis of all these results, we selected a C8 column and an eluent consisting of acetonitrile:0.1M phosphate buffer pH3 (15:85) containing 50mM propylamine hydrochloride.

2 Selectivity of the Proposed Method

Both AMI and HCT were examined for degradation products. The hydrolysis product of HCT is 4-amino-6-chlorobenzene-1,3-disulphonamide (Fig. 3B): this compound did not interfere as it was separated from AMI, HCT and CTZ, the internal standard. The degradation of AMI is not described in the literature. The British Pharmacopoeia mentions only methyl-3,5-diamino-6-chloropyrazine-2-carboxylate, which is an intermediate in the synthesis [19]. We therefore subjected AMI to hydrolysis in alkaline medium (0.1M NaOH) (Fig. 4A). One major degradation compound and several minor ones were formed. The UV spectrum of the major degradation compound was recorded by means of a high-speed spectrophotometric diode-array detector as this compound eluted from the column. UV maxima at 212nm, 270nm and 352nm were found. Compared to AMI (211nm, 285nm and 361nm) a hypsochromic shift occured. This might be due to the loss of the guanidine group resulting in 3,5-diamino-6-chloropyrazine-2-carboxylic acid (Fig. 3A). This compound was prepared by hydrolysing its methyl ester in 0.1M NaOH.

The retention time of the compound and its UV spectrum coincided with the main hydrolysis product of AMI. Fig. 4B shows the separation of AMI from its degradation products and from methyl-3,5-diamino-6-chloropyrazine-2-carboxylate.

Furthermore the proposed method is also specific with regard to the thiazide, the potassium-sparing and the loop diuretics: they all elute later than HCT.

3 Quantitative Determination

For the quantitative determination of AMI and HCT, chlorothiazide (CTZ), the hydrogenation product of HCT, was chosen as internal standard. The other hydrothiazide diuretics were not used since they all elute after HCT [12].

The proposed method was linear for AMI ($r = 0.9998$) over a concentration range from $0.001\,mg\,ml^{-1}$ to $0.050\,mg\,ml^{-1}$ and for HCT ($r = 1.0000$) over a concentration range from $0.010\,mg\,ml^{-1}$ to $0.500\,mg\,ml^{-1}$.

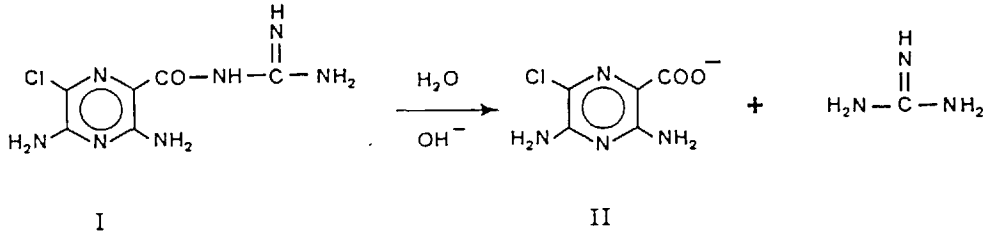
Table IV lists the results of the standard addition-recovery experiments on artificial mixtures at various drug levels.

A recovery of 100.9% (RSD 1.6%) for AMI and 101.9% (RSD 0.90%) for HCT was obtained. The reproducibility of the method was investigated by performing several analyses on a commercial formulation. As the proposed method is quite reproducible (Table V) only a limited number of samples had to be analysed for the other batches.

These results and the results of the analysis of an experimental formulation with different excipients are shown in Table V. Fig. 5 shows a chromatogram of the determination of AMI and HCT, baseline separations were obtained.

The simple extraction procedure with methanol and the fast chromatographic separation (only six minutes for one chromatographic run) result in a short analysis time for both drugs.

A

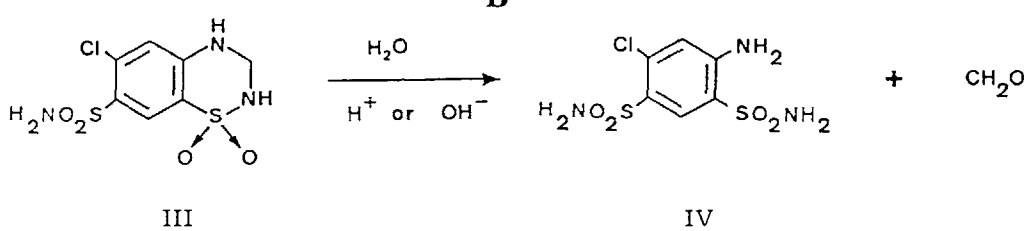


I II

Fig. 3A

Hydrolysis of AMI (I) in 0.1M NaOH to 3,5-diamino-6-chloropyrazine-2-carboxylic acid (II).

B



III IV

Fig. 3B

Hydrolysis of HCT (III) to 4-amino-6-chlorobenzene-1,3-disulphonamide (IV) and formaldehyde.

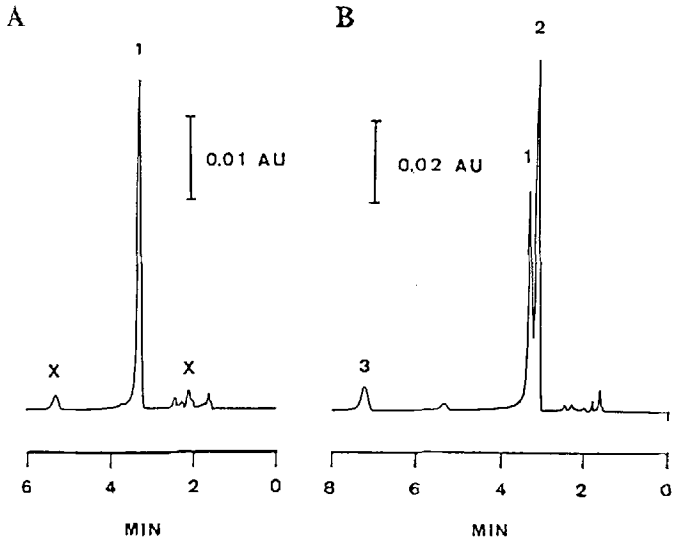


Fig. 4A

Chromatogram of hydrolysis of AMI in mild alkaline medium. (1) is main hydrolysis product. Chromatographic conditions: see Table I.

Fig. 4B

Separation of AMI (2) from 3,5-diamino-6-chloropyrazine-2-carboxylic acid (1) and methyl-3,5-diamino-6-chloropyrazine-2-carboxylate (3).

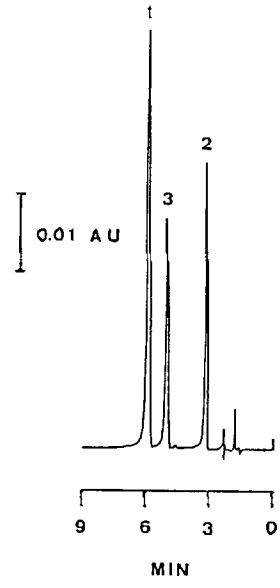


Fig. 5

Chromatogram of simultaneous quantitative determination of HCT (1) and AMI (2). CTZ (3) is internal standard. Chromatographic conditions: see Table I.

Table IV Recovery of AMI and HCT from synthetic tablet mixtures

	Recovery (% of amount added)	
	AMI	HCT
	98.8	101.0
	99.1	101.5
	102.7	102.7
	101.5	102.5
	103.1	102.9
	99.3	102.8
	100.9	100.7
	101.4	101.1
Average	100.9	101.9
RSD, %	1.6	0.90

Table V HPLC analysis of AMI and HCT in two different tablet formulations

	% of label claim	RSD %	n
Commerical Formulation			
Batch 1: AMI	98.2	0.87	10
HCT	101.3	1.6	10
Batch 2: AMI	99.1	–	2
HCT	103.6	–	2
Experimental Formulation			
AMI	97.2	–	2
HCT	102.0	–	2

Conclusion

The proposed HPLC method is a reliable, fast and specific method for the determination of AMI and HCT in the pharmaceutical formulations studied.

References

[1] *P. Bulut, F. Tureli*, Anal. Abstr. **46**, (1984) 1 E 36.
[2] *J. E. Baer, C. B. Jones, S. A. Spitzer*, J. Pharmacol. Exp. Ther. **157**, 472 (1967).
[3] *M. Peterkova, B. Kakak, O. Matousova*, Anal. Abstr. **40**, 4 E 39 (1981).
[4] *K. Reuter, H. Knauf, E. Mutschler*, J. Chromatogr. **233**, 432 (1982).
[5] *M. S. Yip, P. E. Coates, J. J. Thiessen*, J. Chromatogr. **307**, 343 (1984).
[6] *P. P. Koopmans, Y. Tan, C. A. M. Van Ginneken, F. W. J. Gribnau*, J. Chromatogr. **307**, 445 (1984).
[7] *C. H. Spurlock, H. G. Schneider*, J. Assoc. Off. Anal. Chem. **67**, 321 (1984).
[8] *S. Ting*, J. Assoc. Off. Anal. Chem. **66**, 1436 (1983).
[9] *M. A. Korany, H.-J. Franzky*, Sci. Pharm. **51**, 291 (1983).
[10] *R. Weinberger, T. Pietrantonio*, Anal. Chim. Acta **146**, 219 (1983).
[11] *G. N. Menon, L. B. White*, J. Pharm. Sci. **70**, 1083 (1981).
[12] *F. DeCroo, W. Van den Bossche, P. DeMoerloose*, J. Chromatogr. **325**, 395 (1985).
[13] *A. Albert, E. P. Serjeant*, The determination of Ionization Constants, Chapman and Hall, New York, 1984, p. 166–174.
[14] *C. Horváth, W. Melander, I. Molnar*, Anal. Chem. **49**, 142 (1977).
[15] *A. Wehrli, J. C. Hildenbrand, H. P. Keller, R. Stampfli, R. W. Frei*, J. Chromatogr. **149**, 199 (1978).
[16] *B. A. Bidlingmeyer, S. N. Deming, W. P. Prince, J. B. Sachok, M. Petrusek*, J. Chromatogr. **186**, 419 (1979).
[17] *A. Sokolowski, K.-G. Wahlund*, J. Chromatogr. **189**, 299 (1980).
[18] *B. A. Bidlingmeyer, J. D. Del Rios, J. Korpi, J. N. Little*, Advances in Chromatography, 1981, p. 437.
[19] *E. J. Cragoe Jr.*, C. A. **62**, 14698f. (1965).

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