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CARRIER GAS FLOW-RATE AND INJECTION SYSTEM IN CAPILLARY GAS CHROMATOGRAPHY OF UNCONJUGATED AND GLYCINE-CONJUGATED BILE ACIDS

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SUMMARY

The first description of successful capillary gas chromatography of intact glycine-conjugated bile acid derivatives used an automatic solid injection system and required very high carrier gas flow-rates (approximately 20 ml/min) to obtain satisfactory peak shape Peak heights of unconjugated bile acid derivatives using this injection system and the low flow-rates (1–2 ml/min) usually used for such gas chromatographic analyses were very susceptible to small changes in flow-rate This system has been re-examined in an attempt to explain this anomalous behaviour An alternative injection system, the all-glass dropping needle, was also investigated No explanation for the very high carrier gas flow-rates required when using the automatic solid injection system was found The dropping needle injection system, however, produced excellent separation of bile acids and their glycine conjugates as dimethylethylsilyl ethers–methyl esters on non-polar wall-coated capillary columns using normal carrier gas flow-rates of 1–2 ml/min It is clear that the automatic solid injection system originally used has some problem associated with it which can only be overcome, in the case of bile acids and their glycine conjugates, by increasing the carrier gas flow-rate to very high levels

INTRODUCTION

In a recent publication [1] the direct analysis of glycine-conjugated bile acids without hydrolysis of the glycine moiety was described using capillary column

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gas chromatography (GC) Response and peak shape for all the bile acid methyl ester derivatives examined [dimethylsilyl, dimethylethylsilyl (DMES), trimethylsilyl (TMS) and dimethyl-*n*-propylsilyl ethers of unconjugated and glycine-conjugated bile acids] were found to be dependent on carrier gas flow-rate At flow-rates normally used for capillary GC (2 ml min^{-1}) these derivatives of the glycine-conjugated bile acids gave very broad peaks with reduced response and long retention times Wall-coated capillary columns of thin film thickness (0 12 μm) and very high carrier gas flow-rates (approximately 20 ml min^{-1} hydrogen) were found to be necessary to obtain sharp peak shapes and maximum GC responses Contrary to classically accepted theory [2], increasing carrier gas flow-rate did not cause a deterioration in the resolution of either the unconjugated or glycine-conjugated bile acids, although retention times were decreased At low flow-rates commonly used for GC analysis of unconjugated bile acids, peak heights of bile acids increased sharply with increasing flow-rate We were concerned about these unexpected findings which were contrary to classical chromatography theory and thus indicated the presence of some problem We have therefore undertaken further investigations using the original system but also including another carrier gas, helium, and an alternative injection system in an attempt to find a better chromatographic system and perhaps explain our previous observations

EXPERIMENTAL

Materials

Unlabelled bile acids and their methyl esters were obtained from a number of sources (Koch Light Labs , Colnbrook, U K , Steraloids, Croydon, U K ; Sigma, Poole, U K , and BDH, Poole, U K.) and used without further purification, except 5β-cholanic acid which was purified by recrystallization from ethanol Standard solutions of bile acids were made up to 1 mg ml^{-1} with redistilled methanol and stored at $-15\,^{\circ}C$

Solvents and reagents were of analytical-reagent grade from BDH and were used as supplied, except methanol, hexane and ethanol (absolute alcohol 1000, James Burroughs, London, U K) which were redistilled before use All solvents and reagents used were examined to ensure that they did not contain interfering peaks with GC retention times similar to those of the bile acid derivatives under investigation Dimethylethylsilylimidazole (DMESI) was obtained from Apin (Abingdon, U K), trimethylsilylimidazole (TSIM) was a preparation from Pierce and Warner U K (Chester, U K) All glassware was silanized by soaking overnight in 1% (v/v) dimethyldichlorosilane in toluene, washing with toluene and methanol and drying before use Sonication was carried out for 5 min using an ultrasonic bath (Model T-14, L and R Manufacturing, Kearny, NJ, U S A) Derivatives used for gas chromatography were formed as described previously [1]

Capillary gas chromatography

Capillary GC analysis of bile acids was carried out using several chromatographic systems as follows

(i) A Carlo Erba Fractovap 2900 (Erba Science U K , Swindon, U K) gas chromatograph with a flame ionization detector and equipped with a dedicated solid injection carousel system (Carlo Erba Model 210) of essentially the same design as that described by Shackleton and Honour [3] Both hydrogen and helium were used as carrier gases Chromatography was carried out using fused-silica wall-coated open capillary columns coated with one of two similar non-polar stationary phases [CP Sil 5 (CB), 25 m×0 32 mm I.D , 0 12 μm film thickness, Chrompack, London, U K., or DB1 (CB); 30 m×0 255 mm I D , 0 25 μm film thickness, J and W Scientific, Rancho Cordova, CA, U.S A)

(ii) A Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, Cincinnati, OH, U.S A) with a flame ionization detector and equipped with two types of injection systems (a) all-glass dropping needle injector of the type described by Van den Berg and Cox [4], (b) split–splitless Grob injection system [5] with split ratios of 1 2 and 1 4 Both capillary columns were also used in these systems

Air and make-up hydrogen flow-rates to the jet were 250 and 25 ml min^{-1} respectively Detector temperature was maintained at 300°C Gas flow-rates were measured at ambient temperature at the jet using a bubble flow meter

RESULTS[a]

The effect of carrier gas type and flow-rate on the peak-height ratios and resolution of derivatives of a number of unconjugated bile acids, glycine conjugates and steroid hormone metabolites was examined. The effects of changes in the injection temperature were similarly evaluated

Solid injection system with carousel

The relationship between carrier gas flow-rate (measured at the flame ionization detector jet) and inlet pressure over the range 0–5 kg/cm^2, which was the maximum achievable in this system, is shown in Fig. 1 The effect of carrier gas flow-rate on peak-height response, resolution and peak shape was also examined

Using this injection system, considerably improved flame ionization detector response was observed with increasing hydrogen or helium carrier gas flow-

[a]Abbreviations and trivial names lithocholic acid (LCA), 3α-hydroxy-5β-cholanoic acid, chenodeoxycholic acid (CDCA), 3α,7α-dihydroxy-5β-cholanoic acid, ursodeoxycholic acid (UDCA), 3α,7β-dihydroxy-5β-cholanoic acid, deoxycholic acid (DCA), 3α,12α-dihydroxy-5β-cholanoic acid, cholic acid (CA), 3α,7α,12α-trihydroxy-5β-cholanoic acid The prefix glyco (G) is used for bile acids having a glycine moiety at position C-24

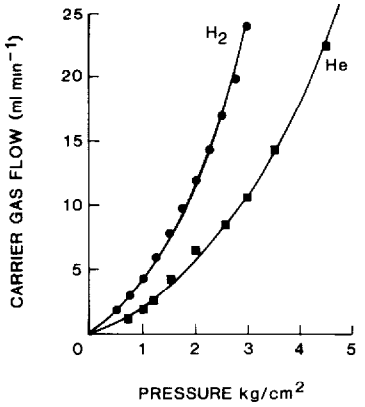

Fig 1 Relationship between carrier gas flow-rate and inlet pressure for hydrogen (●) and helium (■) in the capillary GC system equipped with the Carlo-Erba solid injection carousel

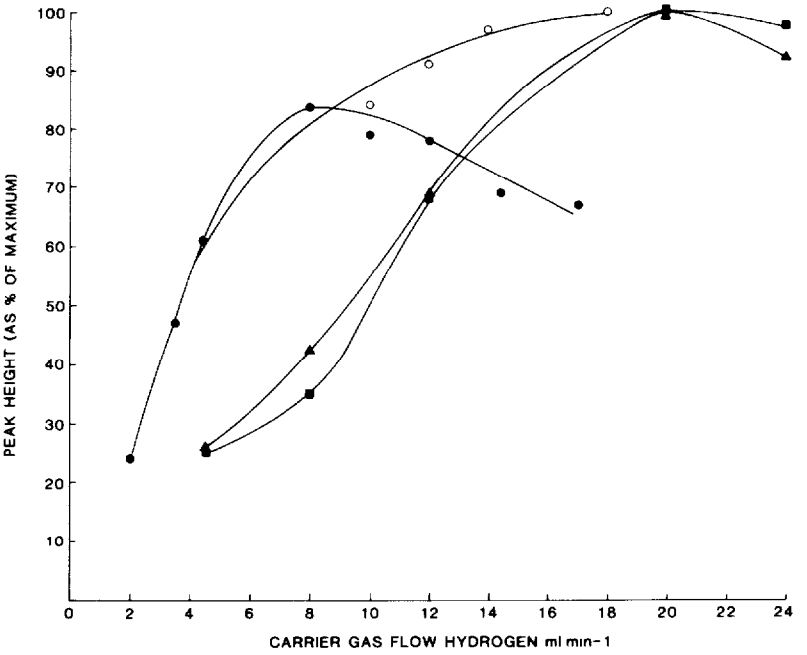

Fig 2 Graph relating peak height as a percentage of maximum to hydrogen carrier gas flow-rate for methyl ester-TMS ethers of glycine-conjugated bile acids using the Carlo-Erba solid injection carousel For glycocholanic acid methyl ester, the auxiliary hydrogen flow-rate to the FID was maintained at 27 ml min^{-1} (●) or reduced as appropriate to permit a constant hydrogen flow-rate of 35 ml min^{-1} to the flame (○) For GLCA (▲) and GCA (■), the auxiliary hydrogen flow-rate to the flame ionization detector was maintained at 27 ml min^{-1} A CP Sil 5 capillary column was used in these investigations

rate Absolute peak heights with constant injection volume and mass of the glycine-conjugated bile acids [glycocholic (GCA), glycochenodeoxycholic (GCDCA), glycodeoxycholic (GDCA) and glycolithocholic (GLCA), all as TMS ethers–methyl esters] increased four- or five-fold with increasing carrier gas flow-rate Maximal response was achieved with a hydrogen carrier gas flow-rate at approximately 15–20 ml min^{-1} (Fig 2) Glycocholanic acid displayed a similar increase in response which maximised at a carrier gas flow-rate of 8 ml min^{-1} with a sharp decrease in peak height at higher flow-rates (Fig 2) Decreasing the auxiliary hydrogen flow-rate to the flame established a plateaux in the response curve for this bile acid (Fig. 2) suggesting high rates of hydrogen flow to the flame disturbs the efficient ionization of this bile acid The peak-height ratio of the derivative of lithocholic acid (LCA), cholic acid (CA) and GCA with respect to cholanic acid increased with increasing hydrogen or helium carrier gas flow-rate, with maximal ratios for GCA at 20 ml min^{-1} (Fig 3) The peak-height ratio of derivatives of unconjugated bile acids with respect to cholanic acid methyl ester responded differently when helium rather than hydrogen carrier gas was used, in that the maximal peak-height ratio was reached at 10 ml min^{-1} with helium carrier gas, whereas 15–20 ml min^{-1} was required with hydrogen carrier gas The phenomenon did not occur with the TMS ethers of the steroids dehydroepiandrosterone, estradiol-17β

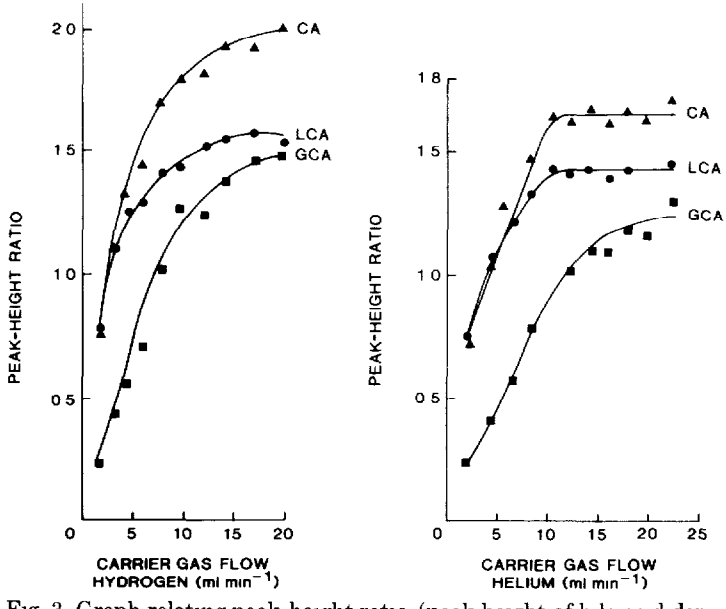

Fig 3 Graph relating peak-height ratio (peak height of bile acid derivative/peak height of cholanic acid methyl ester) to carrier gas flow-rates for methyl ester–TMS derivatives of LCA, CA and GCA using a CP Sil 5 capillary column and the Carlo-Erba solid injection carousel

and etiocholanolone Peak-height ratios of these compounds with respect to the steroid androstan-17-one were constant over a wide range of hydrogen carrier gas flow-rate from 1 to 25 ml min^{-1} (Fig 4) Contrary to classical theory [2], the separation factor (resolution), described by the TMS ether–methyl ester of cholic acid/TMS ether–methyl ester of lithocholic acid retention time

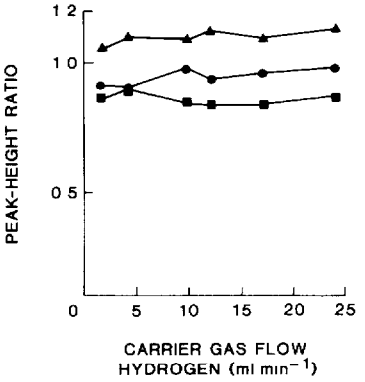

Fig 4 Graph relating peak-height ratio [peak heights of TMS ether derivatives of dehydroepiandrosterone (▲), estradiol-17, (●) and etiocholanolone (■) divided by peak height of internal standard, androstan-17-one] to carrier gas flow-rate using a CP Sil 5 capillary column and a Carlo-Erba solid injection carousel

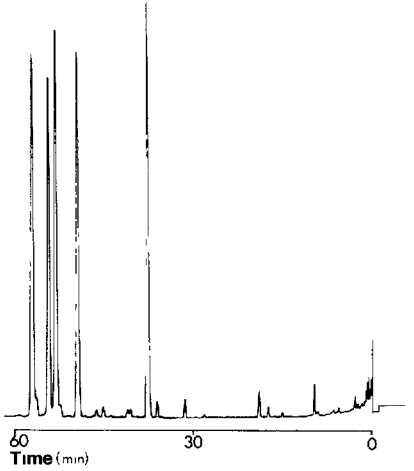

Fig 5 Capillary gas chromatographic separation of glycine-conjugated bile acids as the dimethylethylsilyl ether-methyl ester derivatives using a CP Sil 5 column, automatic solid injection system and high carrier gas flow-rate (22 ml min^{-1}) as described in the text and in ref 1 Methyl ester-DMES derivatives of glycine conjugates are identified, reading from the right to left, as internal standard (glycocholanic acid), GLCA, GDCA, GCDCA and GCA Chromatographic conditions were injection and detector temperature, 300 °C, temperature programmed from 150 to 280 °C at 2 °C min^{-1} and held at 280 °C

ratio did not deteriorate with increasing hydrogen carrier gas flow-rate and may even be marginally improved with increasing flow-rate [1].

Since it was suspected that the glycine-conjugated bile acids might degrade at high temperatures or fail to volatilise at low temperatures, the effect of changes in the injection temperature was examined. Over the range 260–310 °C the peak-height ratio of derivatives of glycine-conjugated bile acid/glycocholanic acid methyl ester remained relatively constant However, below 235 °C the relative peak-height ratio dropped sharply with decreasing injection temperature Considerable tailing of the peaks occurred at 210 °C when compared with peak shape at 280 °C Successful separation of some glycine-conjugated bile acid standards as DMES ethers–methyl esters using this system at the high carrier gas flow-rates (22 ml min^{-1}) found necessary for good peak shape is illustrated in Fig 5

Since the observations described above were unexpected, it was considered possible that the high flow-rates were required to overcome some problem with the solid injection system used Alternative injection systems were therefore examined and these included an all-glass dropping needle and a Grob splitless injector

Solid injection system with all-glass dropping needle

In contrast to the carousel injection techniques used above, the use of the dropping needle injector, in conjunction with CP Sil 5 or DB1 capillary columns, gave reproducible unit responses with equimolar amounts of methyl

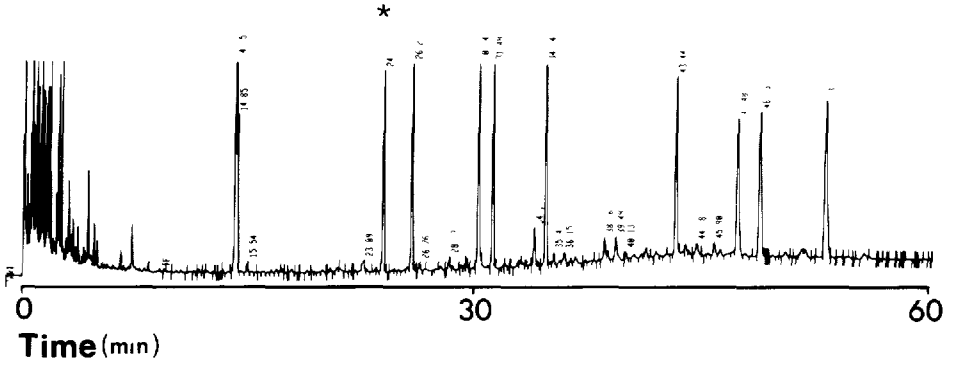

Fig 6 Capillary gas chromatography of bile acids and their glycine conjugates as dimethylethylsilyl ether-methyl ester derivatives on a DB1 column using an all-glass dropping needle injection system as described in the text Helium carrier gas at a flow-rate of 2 ml min^{-1} was used Major peaks are identified, reading from left to right, starting with coprostanol (identified with asterisk) LCA, DCA, CDCA, CA, GLCA, GDCA, GCDCA and mixture of GCA and glycoursocholic acid Chromatographic conditions were injection temperature, 250 °C, detector temperature, 300 °C, temperature programmed from 60 to 225 °C at 5 °C min^{-1}, from 225 to 295 °C at 2 5 °C min^{-1} and held at 295 °C

ester–TMS ether derivatives of LCA, chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), CA and their glycine conjugates at carrier gas flow-rates of 2 ml min^{-1} The effect of increased carrier gas flow-rate could not be studied because of the limitations of the injector system which prevented the use of higher carrier gas flow-rates, but the results achieved with the lower carrier gas flow-rates were comparable with those obtained using the carousel injection technique at higher flow-rates Fig 6 illustrates a typical separation achieved using this injection system with low carrier gas flow-rates

Liquid injection with Grob splitless injection

With the Grob injector, response was more variable – particularly for the unconjugated bile acids At a constant split ratio, but with increased carrier gas and split gas flow-rates, peak-height ratios of derivatives of each of the bile acids (LCA, DCA, CDCA, CA and their glycine conjugates) with respect to 5α-cholestane were constant, but exhibited a wide scatter

DISCUSSION

Optimization of chromatographic conditions for maximal response and reproducibility is essential for successful GC analysis However, selection of optimal conditions may be time-consuming and expensive, involving comparison of injection systems and capillary columns Furthermore, adherence to classical GC theory (Van Deempter plot) [2] would discourage the use of high carrier gas flow-rates found to produce optimal response with the carousel solid injection system [1] used here At such high carrier gas flow-rates the resistance to mass transfer should seriously increase the HETP (height equivalent to theoretical plate) value and hence reduce resolution and response of the compound [2] Our experiments in this and a previous publication [1] have shown that resolution and peak height of the glycine-conjugated bile acid derivatives, and in many cases that of the unconjugated bile acid derivatives, increased with increasing carrier gas flow-rate with maximum response and resolution obtained at carrier gas flow-rates ten to twenty times that usually recommended for capillary GC analysis Increased hydrogen flow-rate to the flame, above that normally recommended, was also shown to increase peak-height response of these compounds but did not fully compensate for low carrier gas flow-rate These observations were made using the automated solid injection system, and the excellent peak response and resolution observed for these compounds using the alternative dropping needle solid injection device suggests that these compounds are particularly susceptible to changes in the method of injection The present study indicates that, since successful chromatography can be carried out at normal carrier gas flow-rates when using alternative injection systems and other steroids examined using the solid injection system do not require such high flow-rates, there must be some problem

with the automatic carousel injection system to which the bile acids and their glycine conjugates are peculiarly susceptible

Inadequate volatilisation, which might be anticipated with compounds of such high molecular weight, or temperature-dependent degradation do not appear to be problems in the successful chromatography of these compounds Above a threshold level of 235 °C, increasing injection temperature did not improve or have deleterious effects on peak-height response Below the threshold level of 235 °C response was markedly decreased suggesting incomplete volatilisation may be a problem at such low temperatures

The choice of internal standard may also have an additional and significant effect on accuracy of quantitation In our studies cholanic acid and glycocholanic acid were chosen as internal standards Cholanic acid is a commonly used internal standard for bile acid mixtures Both compounds are structurally similar to their respective bile acid groups, require methylation and elute close to the compounds of interest on the GC columns used

Fig 2 shows that the glycocholanic methyl ester peak height is affected by the carrier gas flow-rate in a similar fashion to the derivatized glycine-conjugated bile acids In quantitative analysis this might be expected to compensate for changes in carrier gas flow-rate, since the ratio of bile acid to internal standard should remain constant However, Fig 3 shows that the ratio increases despite this compensatory effect Furthermore, on the steep slope of the curve (2–10 ml min^{-1} carrier gas flow-rate) small changes in carrier gas flow-rate will cause significantly increased variability in quantitation It is important to note that this observation applies to both conjugated and unconjugated bile acids derivatives Bile acids are commonly analysed by GC [6] and the need to choose the injection system carefully to avoid the problems described here has not previously been reported

The investigations described in this paper do not provide a complete explanation for the earlier observations but at least provide a better GC system for this analysis, which since it is carried out at normal carrier gas flow-rates, can be linked if required to a mass spectrometer A number of different parameters in the original injection system may have affected the resolution and peak-height response observed the type of injection system used and its geometry in relation to the column, temperature of injection, and carrier gas flow-rate and type of carrier gas It would appear that the glycine-conjugated bile acid (and to a lesser extent unconjugated bile acid) derivatives are particularly suspectible to the method of injection and this may be the reason why successful chromatography of these conjugated compounds has only recently been described [1]

ACKNOWLEDGEMENTS

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REFERENCES

1 J M Street, D J H Trafford and H L J Makin, J Lipid Res , 27 (1986) 208
2 C G Horvath, in B M Mitruka (Editor), Gas Chromatographic Applications in Microbiology and Medicine, Wiley, New York, 1975, Ch 2, p 27
3 C H L Shackleton and J W Honour, Clin Chim Acta, 69 (1976) 267
4 P M J Van den Berg and T P H Cox, Chromatographia, 5 (1972) 301
5 K Grob and K Grob, J High Resolut Chromatogr Chromatogr Commun , 1 (1978) 57
6 J M Street, D J H Trafford and H L J Makin, J Lipid Res , 24 (1983) 491