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# The Use of On-Line Liquid Chromatography/Mass Spectrometry and Stable Isotope Techniques for the Identification of Budesonide Metabolites

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**A moving belt interface was used to identify budesonide metabolites, obtained from rat and mouse liver incubations, by liquid chromatography/mass spectrometry (LC/MS). The metabolites were separated on a small-bore $C_{18}$ column with an ethanol/water gradient as mobile phase at a flow rate of 0.2 ml $min^{-1}$. A spray device was used for deposition of the aqueous solvent on to the belt. Chemical ionization mass spectra were obtained with methane as the reagent gas. Deuterium-labelled budesonide, which was used to facilitate metabolite identification by the isotope cluster technqiue, was found to be slightly separated from the unlabelled analogue on the LC column. Incubations were also performed under $^{18}O_2$ to elucidate the mechanism of a new metabolic pathway (16α,17α-acetal splitting) and to confirm the oxidative nature of reactions leading to hydroxylated metabolites. The moving belt LC/MS technique afforded higher sensitivity, and gave more abundant $MH^+$ ions of the compounds studied, than previously found by direct probe mass spectrometry. Phthalate ester background, partly from the polymide belt, complicated the identification of minor metabolites.**

## INTRODUCTION

In metabolic and pharmacokinetic studies the qualitative and quantitative analyses of synthetic corticosteroids represent a considerable problem owing to the functional group complexity and thermal lability of these compounds. Gas chromatography (GC) has been widely used in the steroid field,[1] but the technique requires derivatization of the oxo and hydroxyl groups, often under rather drastic conditions, and is therefore less suitable for the identification of metabolites of unknown structure and stability. Column liquid chromatography (LC) has gained increasing popularity owing to the mild conditions offered by the technique, and a variety of stationary phases and solvents are available to make the separation of synthetic corticosteroids from endogeneous material possible.[2] The use of mass spectrometry combined with LC adds another dimension to the selectivity with which these compounds can be analysed in biological extracts. Henion and co-workers[3,4] have convincingly demonstrated the utility of direct liquid introduction (DLI) LC/MS for the analysis of corticosteroids in horse urine. For the same purpose, i.e. racehorse drug testing, Houghton *et al.*[5] used LC/MS with a moving belt interface. Although problems with spectrum reproducibility exist,[3,6] on-line LC/MS in many cases represents the best, or only, technique for the detection and identification of corticosteroids in biological samples.

Budesonide (Pulmicort®, Rhinocort®) (Fig. 1) is a topical glucocorticosteroid clinically used in the treatment of asthma and rhinitis.[7] The drug, which is a 1:1 mixture of two epimers with (22R) and (22S) configuration, is characterized by rapid biotransformation in the liver[8,9] and low systemic activity.[10] The two major metabolites of budesonide, 6β-hydroxybudesonide and 16α-hydroxyprednisolone, have previously been identified by direct probe mass spectral analysis of evaporated LC fractions.[11] This off-line LC/MS technique suffered from low sensitivity, probably caused by adsorption of the compounds on to glass surfaces.[5] Since about 10 μg of steroid was required to obtain a mass spectrum of acceptable quality for identification, a number of minor budesonide metabolites eluded identification in our previous work. The use of an LC/MS interface of the moving belt type has now allowed us to perform a more thorough investigation of the biotransformation of the drug. In this paper the advantages and disadvantages of the analytical technique are discussed and exemplified by the identification of budesonide metabolites from rat and mouse liver incubations.

## EXPERIMENTAL

### Chemicals

Budesonide was prepared and resolved into its (22R)- and (22S)-epimers as previously described.[12,13] Deuterated budesonide [($^2H_8$)budesonide; (22RS)-

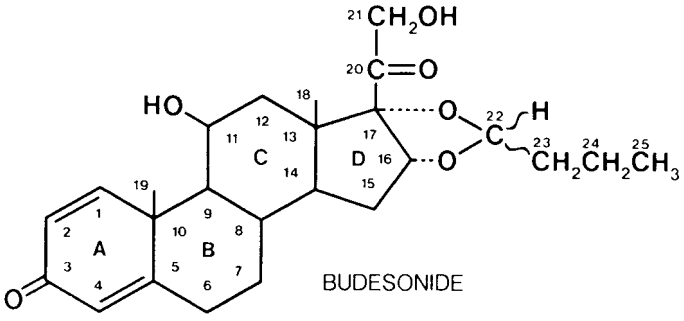


**Figure 1.** Structural formula of budesonide.

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(22, 23, 23, 24, 24, 25, 25, 25-$^2H_8$)-16$\alpha$, 17$\alpha$-butylidenedioxy-11$\beta$, 21-dihydroxypregna-1, 4-diene-3, 20-dione] was prepared from 16$\alpha$-hydroxyprednisolone and $n$-($^2H_8$)butyraldehyde.[14]

$^{18}O_2$ was obtained from the Radiochemical Centre, Amersham, UK. Glucose-6-phosphate, $NADP^+$, glucose-6-phosphate dehydrogenase, phenylmethylsulphonylfluoride (PMSF) and bis($p$-nitrophenyl)phosphate (BPNPP) were obtained from Sigma Chemical Co., St Louis, MO, USA. Sep-pak $C_{18}$ cartridges (Waters Ass., Milford, MA, USA) were conditioned with 5 ml ethanol, followed by 5 ml 0.01 M acetic acid, before use.

Water from a Milli-Q system (Millipore, Molsheim, France) was used for sample preparations and extractions. In the LC/MS experiments glass-distilled water was used. Ethanol was of spectroscopic grade. Ethanol/water mixtures are expressed in v/v terms.

### Liver preparation

Sprague Dawley CR rats (Charles River Breeding Laboratories, St Wilmington, MA, USA) and NMRI mice (Anticimex, Stockholm, Sweden) were used. The animals were killed by decapitation and the livers perfused *in situ* with ice-cold buffer (0.1 M potassium phosphate buffer, pH 7.4, containing 0.2 M sucrose). The livers were then removed, minced, weighed and homogenized with a Teflon pestle (10 strokes) in 2.5 volumes of the same buffer. The homogenates were centrifuged at 9000 $g$ (4 °C) for 20 min and the supernatant fractions stored at −80 °C before use. The protein concentration of the homogenates was determined[15] with bovine serum albumin as standard.

### Incubation medium

The liver 9000 $g$ supernatant fractions were diluted with homogenizing buffer to a protein concentration of 3 mg $ml^{-1}$ and fortified with cofactors (10 mM $MgCl_2$, 40 mM glucose-6-phosphate, 1.8 mM $NADP^+$, and glucose-6-phosphate dehydrogenase, 1.2 units $ml^{-1}$). Incubations were started by the addition of an equimolar mixture of budesonide and ($^2H_8$) budesonide in ethanol to 25–50 ml of incubation medium, giving a final steroid concentration of 10–20 μM in 1% ethanol. The incubations were performed at 37 °C with gentle shaking for up to 2 h under an atmosphere of carbogen (5% $CO_2$ in $O_2$), $^{16}O_2$, or $^{18}O_2$. In all experiments the two epimers of budesonide and ($^2H_8$)budesonide were separately investigated.

### Incubation under $^{18}O_2$

The $^{18}O_2$ experiments were performed in an airtight system (Fig. 2), consisting of two 100-ml vacuum flasks with side arms and rubber septa, a break seal bulb containing 100 ml of $^{18}O_2$, and a manometer. A nitrogen gas supply and a vacuum pump were connected to the system via a three-way valve. The volume of the system, including the break seal bulb, was approximately 350 ml. Liver 9000 $g$ supernatant fraction (25 ml), including cofactors, was added to each flask. In some experiments, aiming at elucidating the mechanism of acetal splitting,

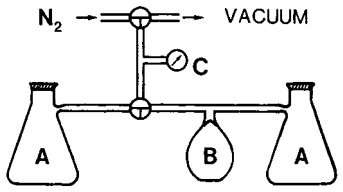


**Figure 2.** Equipment for the $^{18}O_2$ incubation experiment. A = vacuum flask; B = break seal bulb; C = manometer.

esterase inhibitors (PMSF, 1 mM, and BPNPP, 5 mM) were added to one of the flasks. The system was repeatedly evacuated (50 mm Hg) and filled with nitrogen. After the tenth evacuation the bulb seal was broken and $^{18}O_2$ allowed to enter the system. After addition of nitrogen to atmospheric pressure, an ethanol solution of budesonide and ($^2H_8$)budesonide (250 nmol of each) was injected into each flask through the rubber septa.

### Extractions

Incubations were terminated by freezing in dry ice. One volume of ethanol was added, the sample thawed during agitation, and concentrated hydrochloric acid added to a final concentration of 1 M. After centrifugation at 1000 $g$ for 30 min the supernatant fraction was diluted with water to give a final ethanol concentration of 10%. Aliquots (maximum 100 ml) were applied on to Sep-pak $C_{18}$ cartridges, which were washed with 10 ml of 15% ethanol in 0.01 M acetic acid and then eluted with 5 ml of 70% ethanol in 0.01 M acetic acid. The eluates were diluted with 0.01 M acetic acid, reducing the ethanol concentration to 15%, and applied on to new Sep-pak cartridges (maximum 50 ml per cartridge). After washing, as described above, the cartridges were eluted with 5 ml ethanol. The solvent was evaporated at 50 °C under a gentle stream of nitrogen and the residue dissolved in 400 μl of 25% ethanol in 0.01 M acetic acid. Up to 100 μl of the final solution was injected into the LC/MS system.

### LC/MS

Liquid chromatography was performed with a Spectra Physics SP 8700 ternary liquid chromatograph. The chromatographic column (150 × 2.1 mm i.d.) was packed with 5 μm Nucleosil $C_{18}$ (Machery-Nagel, Düren, FRG). Gradient elution was performed with ethanol and water as mobile phase at a flow rate of 0.2 ml $min^{-1}$ (Table 1). The system was allowed to equilibrate for at least 25 min between injections. A Waters Model 440 UV detector (254 nm) was connected between the LC column and a Finnigan LC/MS moving belt interface. The LC effluent was fed to the polymide (Kapton$^R$) belt via a spray device (directed perpendicular to the belt), constructed according to Fig. 3 (cf. Refs 16, 17). Heated nitrogen (170 °C) was used as nebulizing gas. The original belt drive motor, running at fixed speed, was replaced with a variable speed dc motor. The belt was operated at a speed of approximately 3.5 cm $s^{-1}$. The IR heater was operated at full power, the flash vaporizer temperature was kept at 220 °C and the clean-up heater temperature at 270 °C. The interface was connected to

**Table 1. Gradient system for LC/MS analysis of budesonide metabolites. Solvent A: 25% (v/v) ethanol in water. Solvent B: 75% (v/v) ethanol in water**

| Time[a] (min) | Solvent A (%) | Solvent B (%) | Ethanol conc. (%) |
|---|---|---|---|
| 0 | 93 | 7 | 28.5 |
| 10 | 90 | 10 | 30.0 |
| 17 | 65 | 35 | 42.5 |
| 35 | 65 | 35 | 42.5 |
| 40 | 0 | 100 | 75.0 |
| 50 | 0 | 100 | 75.0 |

[a] At a flow rate of 0.2 ml $min^{-1}$ solvent composition lagged about 10 min at the column top, due to large dead volume in the mixing chamber of the liquid chromatographic pump.

a Finnigan 4500 quadrupole mass spectrometer equipped with a Finnigan INCOS data system. Methane was used as chemical ionization (CI) reagent gas at an ion source pressure of 0.5 Torr and an indicated ion source temperature of 150 °C. A clean CI ion volume was installed every day before analysis. Data acquisition was started at time of injection and mass spectra were repetitively recorded between $m/z$ 200 and 550, at a rate of 3 s per scan.

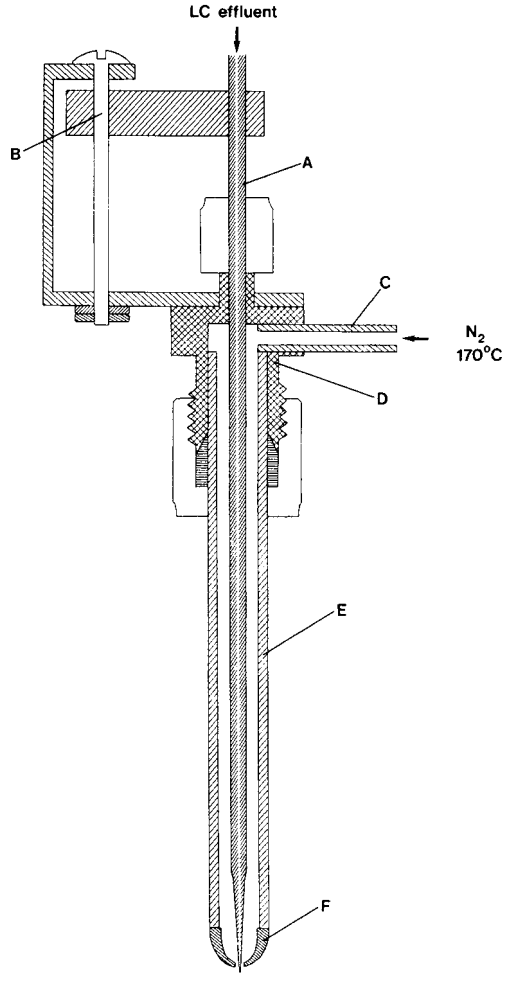


**Figure 3.** Schematic illustration of the spray deposition device. (A) Stainless-steel capillary tubing $\frac{1}{16}$ inch (0.15 mm i.d.); (B) adjustment screw for optimization of spray performance; (C) stainless-steel tubing $\frac{1}{8}$ inch (2 mm i.d.); (D) standard LC column top; (E) stainless-steel LC column tubing $\frac{1}{4}$ inch (4.6 mm i.d.); (F) brass nozzle with approximately 0.6 mm orifice.

## RESULTS AND DISCUSSION

### LC/MS system

Budesonide is extensively metabolized in the liver[8,9] and the various metabolites differ considerably in polarity. For the separation of the metabolites an ethanol–water gradient run on a $C_{18}$ column was found to be more selective than straight phase systems based on amino or nitro columns. However, difficulties exist in the use of aqueous solvents in combination with the moving belt interface (for a review of this interface see Ref. 18). Due to poor wetting of the hydrophobic polyimide belt, the polar solvent is non-uniformly distributed on the belt surface, resulting in distorted peak shapes. In addition, the relatively low volatility of the mobile phases used in reversed phase chromatography poses restrictions on the flow rate. Spray deposition of the solvent on to the belt, with argon[16] or nitrogen[17] as nebulizing gas, has been reported to give much improved performance. In the present study, the use of a similar spray device greatly improved the deposition and evaporation of the aqueous solvent. The chromatographic solvent system used contained up to 72% of water in ethanol, which could be handled by the interface at a flow rate of 0.2 ml $min^{-1}$. Figure 4 shows the separation of budesonide metabolites in an extract from a rat liver incubation. Comparison of the UV trace with the total ion current (TIC) chromatogram shows that the chromatographic integrity is well maintained (peaks were delayed for 21 s in the LC/MS interface). The use of a UV detector connected in series with the LC/MS interface was valuable for structural elucidation of metabolites. Since the UV response of budesonide at 254 nm is due to the $\alpha,\beta$-unsaturated ketone in the A-ring, hydrogenation of this conjugated system will reduce the UV absorptivity. The A-ring reduced metabolites (Fig. 4) could therefore be recognized by their reduced UV response in combination with an increase in molecular weight by 2 or 4 u, relative to budesonide.

### Deuterium labelling

Analytical methods based on mass spectrometry make it possible to use drugs labelled with stable isotopes as tracers in metabolic studies.[19] Incubation of a mixture of deuterium-labelled and -unlabelled drug will create an artificial isotope cluster, which is easily recognized in the mass spectra of the metabolites. This twin ion technique greatly facilitated the detection and identification of minor budesonide metabolites. On the LC column, however, the isotope analogues separated to some extent, the deuterated compounds having a somewhat shorter retention time (Fig. 5). This caused an apparent broadening of the chromatographic peaks, which complicated the search for isotope clusters. To obtain a mass spectrum of the typical twin ion appearance data had to be averaged over the entire chromatographic peak, which reduced the signal-to-noise ratio.

Hydroxylation of the 6$\beta$-position is a major metabolic pathway for budesonide[11] as well as for many other corticosteroids. In the present study we were able to detect another hydroxylated metabolite, in which the

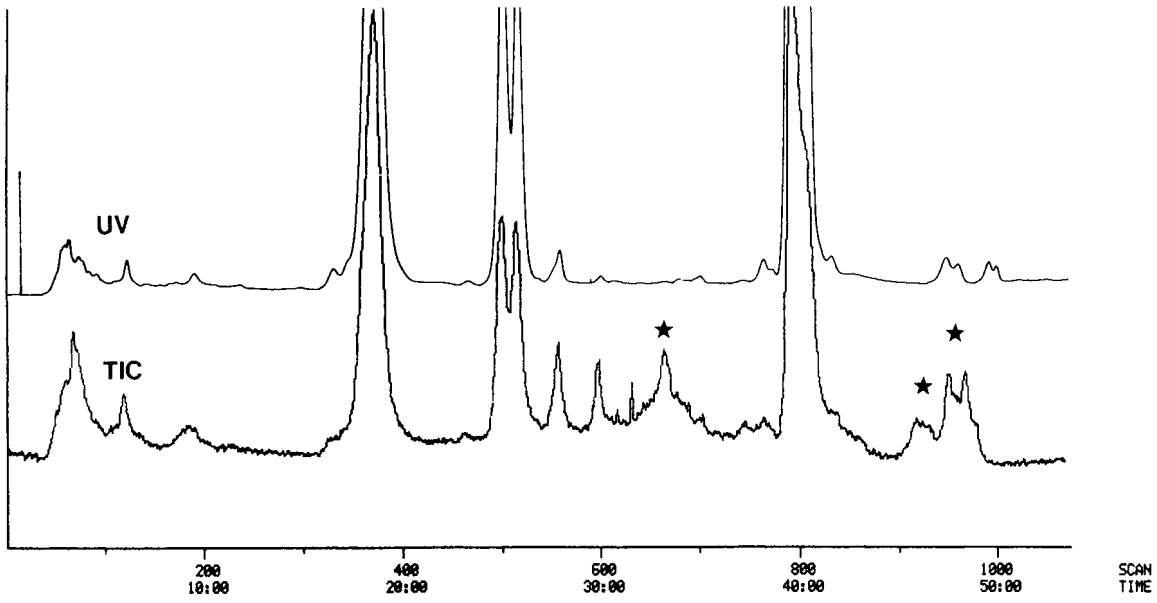


**Figure 4.** UV trace and TIC chromatogram from LC/MS analysis of an extract obtained after incubation of the (22S)-epimer of budesonide and ($^2H_8$)budesonide with rat liver 9000 *g* supernatant fraction. The asterisks indicate A-ring hydrogenated budesonide metabolites with reduced UV response.

deuterated analogue had lost one of its eight deuterium atoms (Fig. 6), indicating biotransformation in the acetal moiety.[21] The spectrum in Fig. 6 also indicates a significant isotope effect in the hydroxylation of the deuterated acetal group. Primary deuterium isotope effects between 1.2 and 11 have been reported for various hydroxylation and dealkylation reactions.[19] In this study, the ratio of *m/z* 447 ($MH^+$ of protium analogue) over *m/z* 454 ($MH^+$ of deuterium analogue) ranged between 5 and 6 in spectra of the acetal hydroxylated metabolite from different incubations.

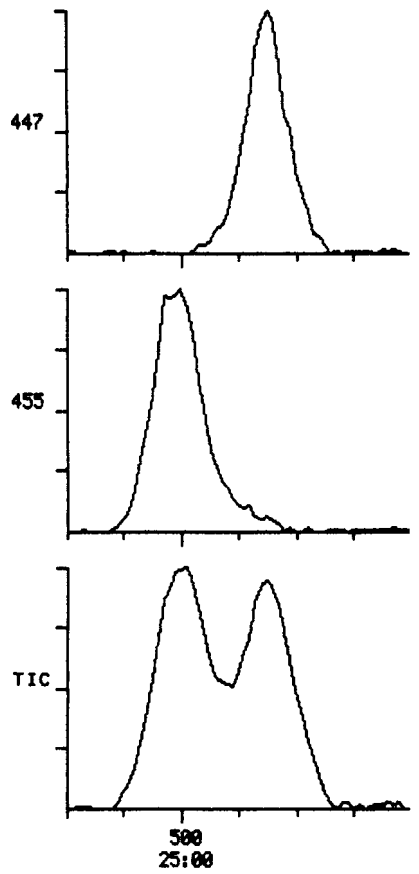


**Figure 5.** Expanded portion of the TIC chromatogram shown in Fig. 4, demonstrating the separation ($\alpha = 1.04$) of deuterium-labelled 6$\beta$-hydroxybudesonide (*m/z* 455) from its unlabelled analogue (*m/z* 447).

## Incorporation of $^{18}O$

Metabolic cleavage of the 16$\alpha$,17$\alpha$-acetal substituent has not been reported for other corticosteroids. This biotransformation pathway is unique for (22R)-budesonide,[11] leading to the formation of 16$\alpha$-hydroxyprednisolone, one of the major metabolites. Cleavage of the acetal side chain of ($^2H_8$)budesonide results in loss of the deuterium label, and mass spectra of metabolites formed by this route did not display the characteristic isotope pattern. We have previously shown[11] that the acetal splitting requires NADPH, is inhibited by SKF 525A, and is catalysed by liver microsomal enzymes, indicating an oxidative reaction. To investigate the mechanism of this reaction, incubations were performed under $^{18}O_2$. The experiment revealed that $^{18}O$ was not incorporated into 16$\alpha$-hydroxyprednisolone. The oxygen isotope could be traced, however, in an intermediary ester, 16$\alpha$-butyryloxyprednisolone, which could be isolated if esterase inhibitors were present in the incubation medium.[20] In the presence of esterase activity the intermediate was rapidly hydrolysed to butyric acid and 16$\alpha$-hydroxyprednisolone. The incubations under $^{18}O_2$ were also used to confirm that the postulated hydroxylated metabolites were formed by an oxidative reaction. The oxygen isotope was found to be incorporated in all of these metabolites, as indicated by an increase in molecular weight by 2 u, relative to the corresponding metabolites formed under $^{16}O_2$. Figure 7 shows the mass spectra of 6$\beta$-hydroxybudesonide formed in incubations under $^{16}O_2$ or $^{18}O_2$.

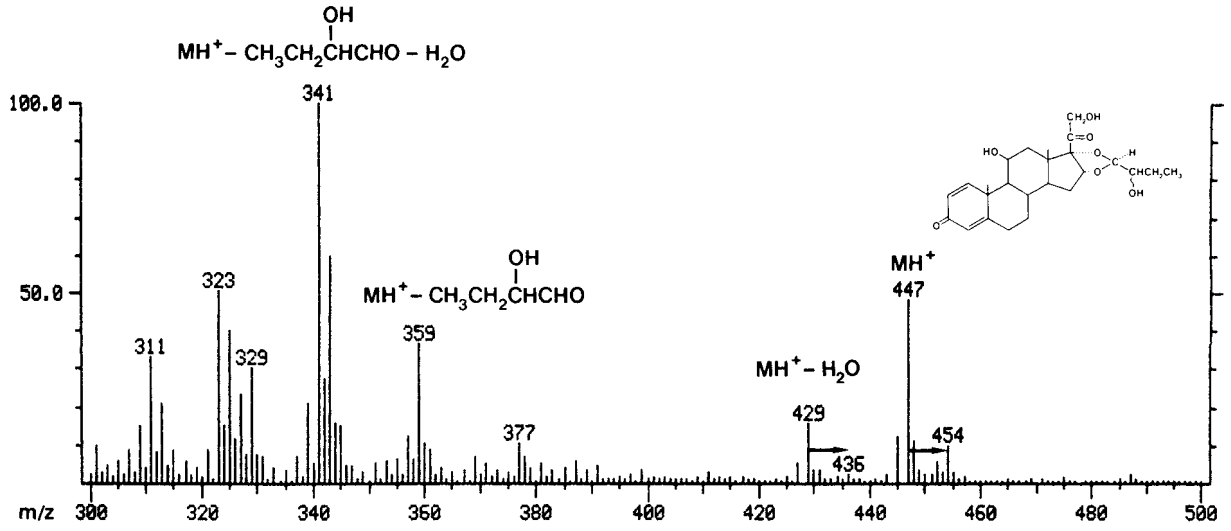


**Figure 6.** CI($CH_4$) mass spectrum of a budesonide metabolite hydroxylated in the acetal group. The sample was obtained after incubation of the (22S)-epimer of budesonide and ($^2H_8$)budesonide with mouse liver 9000 *g* supernatant fraction. Arrows indicate deuterium-containing ions.

## Advantages of on-line LC/MS

On-line LC/MS, as well as GC/MS, allows mass spectra to be repetitively recorded during the chromatographic run. Ions of interest can later be selected and plotted as continuous ion chromatograms. An example of the ability of such selected ion current profiles to 'resolve' closely eluting compounds is shown in Fig. 8. The peak in the TIC chromatogram was first believed to be due entirely to unchanged budesonide (*m/z* 431 and 439), but a mass spectrum from this region showed peaks at *m/z* 429 and 437 as well. When selected ion current

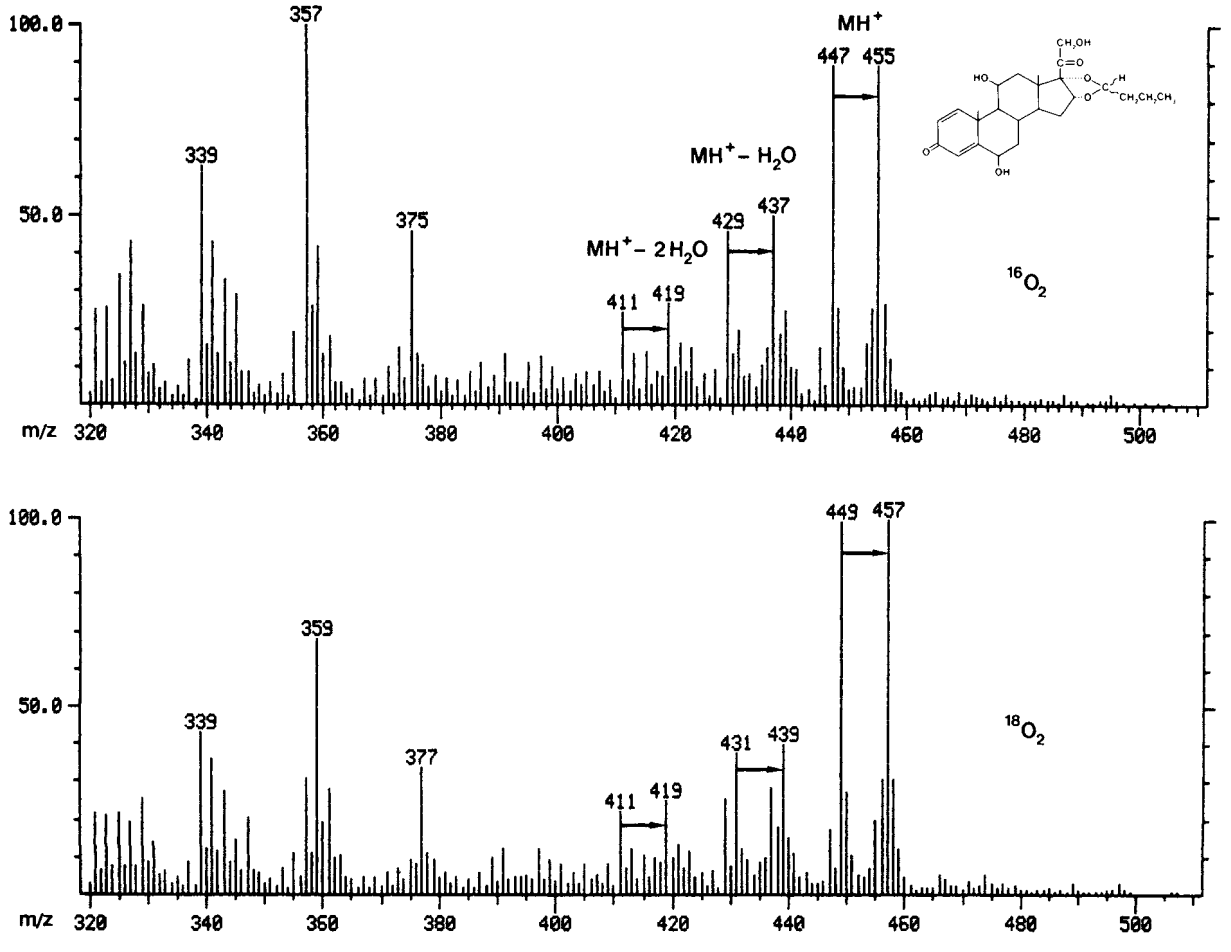


**Figure 7.** CI($CH_4$) mass spectra of 6$\beta$-hydroxybudesonide formed by incubating the (22R)-epimer of budesonide and ($^2H_8$)budesonide with mouse liver 9000 *g* supernatant fraction under $^{16}O_2$ or $^{18}O_2$. Arrows indicate deuterium-containing ions.

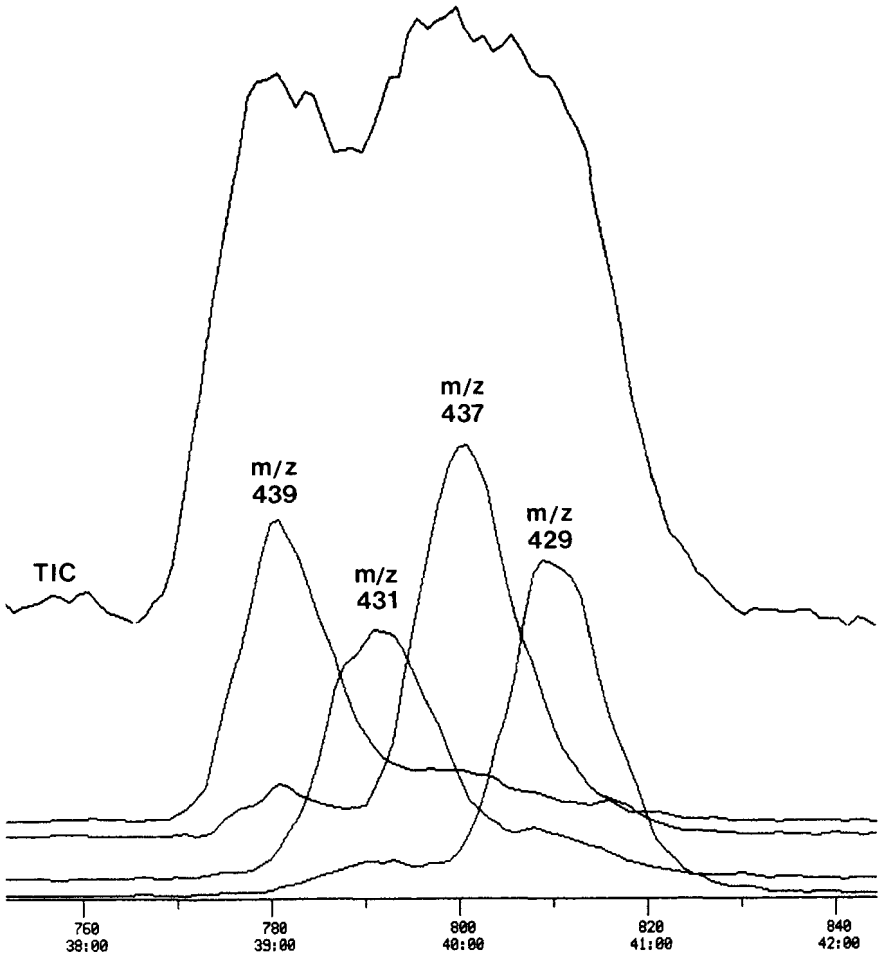


**Figure 8.** Expanded portion of a TIC chromatogram and selected ion current profiles of the $MH^+$ ions corresponding to budesonide (*m/z* 431) and $\Delta^6$-budesonide (*m/z* 429) plus their deuterium-labelled analogues (*m/z* 439 and 437, respectively). The sample was obtained after incubation of the (22R)-epimer of budesonide and ($^2H_8$)budesonide with rat liver 9000 *g* supernatant fraction.

chromatograms of these ions were plotted it became apparent that an unknown compound eluted very close to budesonide. This metabolite was overlooked in our previous work on budesonide metabolism[11] because it was not chromatographically separated from budesonide, nor was it detected in the narrow LC fractions collected at the retention time of budesonide for direct probe mass spectral analysis. The compound was later identified as $\Delta^6$-budesonide by comparison with authentic material.[21]

Corticosteroids with a free hydroxyl group in the 17-position are difficult to analyse by mass spectrometry due to thermal decomposition into the corresponding 17-keto steroid during evaporation.[6,22] 16$\alpha$-Hydroxyprednisolone, formed from budesonide by cleavage of the 16,17-acetal group, contains free hydroxyl groups in both 16- and 17-positions. In our previous investigation on budesonide metabolism by direct probe mass spectral analysis[11] only a weak (2%) $MH^+$ ion of 16$\alpha$-hydroxyprednisolone was obtained, even if relatively large amounts (more than 10 μg) were introduced into the ion source. Analysis of comparable amounts by the LC/MS system described in this paper resulted in a much improved spectrum with a relative abundance of the $MH^+$ ion of up to 60%. We observed, however, that the amount of sample had a strong influence on spectrum quality, as discussed by Cairns *et al.*[6] For many of the metabolites a threshold, regarding sample amount, seemed to exist. Below the threshold thermal decomposition was extensive. This effect may explain why spectra obtained from solutions applied with a syringe directly on to a short distance of the belt have been reported to exhibit more relative molecular mass information than if the same amount of sample was distributed over a longer distance via the LC column.[18,23]

## Phthalate background

Throughout this study a significant phthalate ester background (probably bis(2-ethylhexyl)phthalate, molecular weight 390) was observed, which made the interpretation of mass spectra from low-level components more difficult. The background was in part due to the Kapton belt, as indicated by the high background found with a new belt. A new belt was therefore conditioned by running it overnight at high clean-up heater temperature before use. Phthalate background from a polyimide belt was also reported by Beattie *et al.*[24] but the problem could be reduced by using ammonia as the CI reagent gas. Unfortunately, the evaporation of phthalate esters from the belt, in the present study, seemed to be favoured by the presence of other components. The intensity of background ions changed concurrently with the total

ion current, which made it difficult to obtain 'clean' spectra of the metabolites through background subtraction.

## CONCLUSION

We have found on-line LC/MS with a moving belt interface to be a valuable technique for the identification of budesonide metabolites, superior to the off-line technique we previously used. Positive ion chemical ionization (PCI) with methane as the reagent gas generally gave abundant (>50%) $MH^+$ ions of metabolites less polar than 16$\alpha$-hydroxyprednisolone. With ammonia as reagent gas some metabolites gave spectra which were difficult to interpret, probably due to ammonium ion adduct formation. We found that negative ion chemical ionization (NCI) mass spectra of budesonide metabolites often lacked molecular weight information, a shortcoming also reported for NCI spectra of other corticosteroids.[3,5] Due to its high sensitivity NCI may be the method of choice for the detection of low levels of targeted compounds, but it seems to be unsuitable for the identification of corticosteroids of unknown structure. In the positive ion mode $MH^+$ ions have been obtained for labile compounds such as dexamethasone and betamethasone with both the DLI technique[25] and the moving belt interface.[6] Promising results with the thermospray interface have been reported for betamethasone,[26] and this technique will undoubtedly facilitate the analysis of corticosteroids in the future.

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