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Simultaneous determination of beclomethasone, beclomethasone monopropionate and beclomethasone dipropionate in biological fluids using a particle beam interface for combining liquid chromatography with negative-ion chemical ionization mass spectrometry

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ABSTRACT

A new simple and sensitive assay has been developed for the simultaneous quantitative measurement of beclomethasone dipropionate and its hydrolysis products in human plasma and urine. Beclomethasone 17,21-dipropionate, beclomethasone 17-monopropionate, beclomethasone and the internal standard, dexamethasone 21-acetate, were measured by combined liquid chromatography and negative-ion chemical ionization mass spectrometry with methane as the reagent gas. A particle beam interface from Hewlett Packard was used. Under mild operating conditions, abundant and stable characteristic high-mass ions were generated in the ion source of the mass spectrometer by a resonance electron-capture mechanism. The fast extraction procedure requires 1 ml of plasma or urine, and the quantification limit of the method is 1 ng ml^{-1} for the three tested compounds.

INTRODUCTION

Beclomethasone 17,21-dipropionate (BDP; I), a potent anti-inflammatory drug, is a synthetic corticosteroid used in the treatment of asthma and allergic rhinitis. More recently, it was successfully used to treat patients suffering from inflammatory bowel disease involving the terminal ileum and the ascending colon. *In vivo*, BDP is extensively hydrolysed to the 17-monopropionate ester (BMP; II) and finally to the corresponding alcohol (BOH; III) (Fig. 1). Owing to the high effectiveness of the drug, the usual therapeutic dose of BDP is very low

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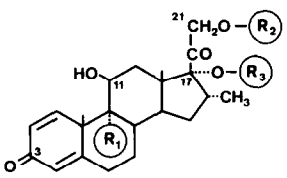

	R_1	R_2	R_3	m.w
BDP I :	Cl	C_2H_5CO	C_2H_5CO	520
BMP II :	Cl	H	C_2H_5CO	464
BOH III:	Cl	H	H	408
I.S :	F	CH_3CO	H	434

Fig. 1. Structural formulae of BDP, BMP, BOH and the internal standard.

(1–5 mg/day), and the resulting plasma and urine concentrations are in the nanogram to subnanogram range per millilitre. Consequently, a sensitive and specific method is required for the simultaneous quantitative analysis of I, II and III in body fluids, and the assay must be sufficiently robust for routine use in pharmacokinetic studies.

It is well known that the qualitative and quantitative analysis of synthetic corticosteroids is a major challenge owing to the thermal lability of these compounds. Gas chromatography (GC) has been widely used in the steroid field but, to overcome the problems encountered during the GC analysis of these compounds, the derivatization of the oxo and hydroxyl groups is required, often under rather drastic conditions. Many derivatives have been previously investigated and some of them were found to be suitable for the GC or gas chromatographic–mass spectrometric (GC–MS) determination of the corticosteroïds [1–13]. However, the derivatization procedure is time-consuming and often leads to multiple products, owing to incomplete reactions.

Several radioimmunoassays (RIAs) have also been developed, but the cross-reactivity with structurally similar compounds, the poor reproducibility and some false determinations are serious restrictions on the use of RIA techniques [14–16].

In contrast to RIA, the high-performance liquid chromatographic (HPLC) technique offers the advantage of sufficient specificity under mild operating conditions [17–24]. Unfortunately, owing to the lack of sensitivity, low levels of corticosteroids cannot be accurately measured with HPLC methods. This problem can be overcome if a powerful detector, such as a mass spectrometer, is coupled to the LC system. Some authors have clearly demonstrated that on-line LC–MS represents in may cases the only technique for the identification of corticosteroids in complex biological matrices [25–28]. A great variety of on-line interfaces have been designed for the efficient transport of the solute from the HPLC system to the ion source of the mass spectrometer, using conventionnal flow-rates (0.3–1 ml min^{-1}) with minimal sample thermal decomposition and chromatographic peak distortion. Recent reports have shown that the monodisperse aerosol generation interface for combining liquid chromatography with mass spectrometry (MAGIC-LC–MS) provides quantitative and linear response and gives detection limits in the nanogram to subnanogram range [29–32]. Up to

now, no simultaneous quantitative assay of beclomethasone and its esters in biological samples has been previously published. We have developed a new LC–MS technique using the particle beam interface commercially available from Hewlett Packard. The negative-ion chemical ionization (NICI) MS detection with methane as the reagent gas allows the determination of trace amounts of I, II and III in plasma and urine samples down to 1 ng ml^{-1}. The feasibility of this simple and sensitive assay was exemplified by preliminary results on the quantitative analysis of I, II and III in biological extracts following oral or rectal administration of I to healthy volunteers.

EXPERIMENTAL

Chemicals

Chemical standards (beclomethasone 17,21-dipropionate, beclomethasone 17-monopropionate, and beclomethasone alcohol) were kindly supplied by Chiesi Farmaceutici Laboratories (Parma, Italy). Dexamethasone 21-acetate (internal standard; I.S.) was purchased from Sigma (L'isle d'Abeau Chesnes, France). Methanol (Carlo Erba, Milan, Italy) and water (Osi, Paris, France) were of HPLC grade. Dichloromethane was obtained from Merck (Darmstadt, Germany) and was used without further purification. The buffer (pH 7) was commercially available (Merck). The mobile phase was filtered under vacuum through a 0.20 μm pore size filter (Millipore, Bedford, MA, U.S.A.) and then continuously purged with helium during analysis in order to remove dissolved gases.

All glassware was cleaned with a mechanical scaling brush, then left overnight in CrO_3–H_2SO_4, and finally rinsed with doubly distilled water. The PTFE caps of the tubes were also carefully cleaned to avoid any subsequent sample contamination.

Standard curves

Stock solutions of I, II, III and the I.S. were prepared by dissolving each pure reference compound in ethanol to an initial concentration of 0.1 mg ml^{-1}. Secondary standard solutions, obtained by appropriate dilutions in ethanol, were protected from light by aluminium foil and stored at 4°C until used.

Aliquots of 1 ml of drug-free human plasma (or urine) were fortified with 25 μl of a 1 μg ml^{-1} I.S. solution in ethanol and various amounts of I, II and III ranging from 1 to 40 ng ml^{-1}. The blank samples were prepared in a similar way by spiking 1 ml of control plasma (or urine) only with the I.S. solution.

Extraction from plasma and urine samples

Plasma or urine (1 ml) samples, spiked as stated above with the I.S. solution, were placed into 20-ml screw-capped tubes. Plasma samples were diluted with 2 ml of water and urine samples were buffered with 2 ml of a buffer (pH 7) in order to standardize the pH of the samples. After a brief mixing, 9 ml of dichloro-

methane were added to each tube. Extraction was carried out for 15 min using a reciprocating shaker, and the tubes were centrifuged at 2200 *g* for 5 min. The upper phase was removed under vacuum; the remaining organic layer was then decanted into a 10-ml Quickfit glass tube and evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 120 μl of mobile phase, and 20 μl were injected into the HPLC system.

Precision, accuracy and limit of detection

To assess the precision and accuracy of the method in plasma samples, repeatability assays were carried out at four different concentrations (2, 5, 10 and 40 ng ml^{-1}). The spiked plasma samples were analysed the same day by the same analyst and, for each concentration level, coefficients of variation and mean percentages of error were calculated.

The limit of detection (LOD) was defined as the lowest detectable concentration yielding a signal significantly higher than that of the blank control specimens. First, to calculate the LOD of the method, a repeatability assay was performed with ten blank plasma samples. The mean signal ($\bar{y}_{bl}$) eventually observed at the retention times of I, II and III, and the associated standard deviation (S_{bl}), were then used to calculate a quantification limit.

HPLC–MS analysis

Liquid chromatography was performed with an unmodified Waters M510 pump (Waters Assoc., Saint-Quentin en Yvelines, France) equipped with a 20-μl Valco external loop injector (Touzart & Matignon, Vitry sur Seine, France). The chromatographic column (100 mm × 4.6 mm I.D.) was packed with 3-μm particle size Microspher C_{18} (Chrompack, Les Ulis, France). The elution was carried out with methanol–water (75:25, v/v) at a flow-rate of 0.4 ml min^{-1}.

The HPLC system was connected to a Hewlett Packard 59980 A particle beam interface. The LC effluent was converted into an aerosol in a pneumatic nebulizer, where helium was introduced coaxially at a flow-rate of *ca.* 1 l min^{-1}. The aerosol sprayed into a desolvation chamber, in which the pressure was maintained at *ca.* 200 Torr and the temperature was held at 55°C. Finally, a beam of virtually solvent-free particles was introduced into the ion source of a Hewlett Packard 5988 mass spectrometer. The MS system was operated in the NICI mode with an electron energy of 100 eV, an emission current of 300 μA and an ion source temperature of 250°C. Prior to the analysis, the instrument was tuned in the NICI mode, using the fragments *m/z* 414, 595 and 633 from the perfluorotributylamine calibrant gas.

The reagent gas (methane) was admitted via a gas-flow controller to an indicated ion source pressure of *ca.* 0.8 Torr. The NICI mass spectra of I, II, III and the I.S. were recorded during an HPLC run by scanning repetitively the quadrupole mass filter every 1.1 s from *m/z* 100 to 550. Quantitative determination was performed by focusing the instrument in the selected-ion monitoring (SIM) mode

in order to measure the fragments *m/z* 505, 449 and 393 for I, II and III, respectively, and *m/z* 374 for the I.S., with a dwell time of 300 ms for each mass range.

Drug administration

Nine healthy male volunteers (21–28 years old) were found to be in good health through medical history, physical examination and routine laboratory profiles. None of the subjects received medication for at least ten days prior to the study. Each subject received two single rectal administrations (enemas with 3 mg of BDP and suppositories with 5 mg of BDP) and a single oral dosing (tablets with 5 mg of BDP). This study was conducted according to an open three-way cross-over protocol, with a wash-out period of one week between two consecutive administrations. Blood samples (5 ml) were collected in heparinized tubes at 0, 1, 2, 4, 8, 12 and 24 h after the administration of the drug. The plasma was separated by centrifugation and stored frozen at −20°C until analysis.

Urine samples were collected in fractions corresponding to the following time intervals: 0–2 h, 2–6 h, 6–12 h and 12–24 h.

RESULTS AND DISCUSSION

HPLC–MS analysis

For a good chromatographic separation of the four compounds, different stationary and mobile phases were tested, taking into account that the particle beam interface can operate at mobile phase flow-rates as high as 1 ml min^{-1} but is optimized at 0.4 ml min^{-1}. As a result, a 100 mm × 4.6 mm I.D. column packed with a highly deactivated stationary phase was found to be optimal for this experiment. The four compounds were well resolved without broadening and under our analytical conditions, the retention times were 3.9 min for III, 4.4 min for the I.S., 5.4 min for II and 8.9 min for I.

In order to optimize the performance of the particle beam interface, the nebulizer position and helium gas flow-rate must be carefully adjusted. The nebulizer adjustment is dependent on the mobile-phase, and the shape of the spray is an important operating parameter. Even if a maximum signal is observed when droplets or a stream form on the end of the nebulizer, further results may be erratic.

The first- and second-stage skimmers in the momentum separator are subject to occasional clogging when high proportions of volatile buffers are added into the mobile phase. In this assay, the methanol–water mixture used as the mobile phase overcomes this major problem, and the two skimmers were operated over a very long period of time without clogging. Moreover, even with a relatively high proportion of an aqueous mobile phase, the temperature of the desolvation chamber was maintained at a low temperature (*ca.* 55°C). Thus, when dealing with thermally labile compounds, the particle beam interface is preferable to some other transport systems, such as the moving-belt interface, in which the

temperature of the flash vaporizer is rapidly raised to 250°C [25,26]. Otherwise one can observe that difficulties exist with the use of aqueous solvents in combination with the moving-belt interface. The lack of a gaussian-shape elution profile is principally due to the fact that the solvent is not uniformly distributed on the polyimide belt.

Following the injection of each pure reference standard (20 ng), the NICI mass spectra of I, II, III and the I.S. were obtained by on-line LC–MS with methane as the reagent gas. The different mass spectra exhibit characteristic ions in the high-mass region as shown in Fig. 2a–d. The molecular anions of I (*m/z* 520), II (*m/z* 464) and III (*m/z* 408) are observed for the three corticosteroids, with a relative intensity varying between 25 and 10%. Under NICI conditions, the dominant mechanism is a resonance electron-capture process. In the mass spectra of the two

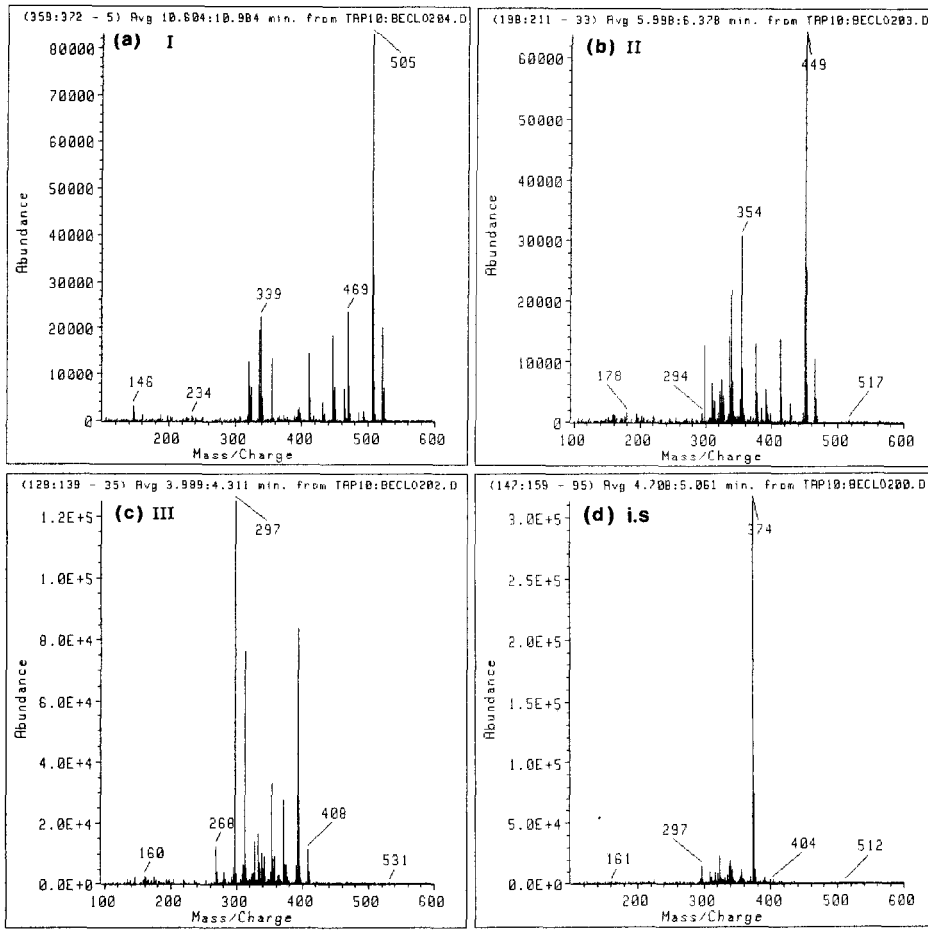

Fig. 2. Negative-ion chemical ionization mass spectra of (a) BDP (I), (b) BMP (II), (c) BOH (III) and (d) the I.S., obtained by HPLC–MS with methane as the reagent gas.

esters I and II, the base peaks are measured at *m/z* 505 and 449, respectively, and these fragments are derived by loss of a methyl radical from the molecular anions. The $[M-(CH_3)]^-$ ion of the corresponding alcohol III (*m/z* 393) is present with a 60% relative intensity, and the base peak is measured at *m/z* 297. Further losses of small moieties give rise to other abundant diagnostic fragment ions at *m/z* > 300. The mass spectrum of the I.S. is simpler: the molecular anion (*m/z* 434) is virtually absent, and the predominant ion at *m/z* 374 can be accounted for by cleavage of the C-21 ester function.

Precision and accuracy

The overall recoveries of I, II and III, following extraction from biological samples, varied from 85 to 90% when *ca.* 90–95% of the dichloromethane phase was available for evaporation. The coefficients of variation (C.V.) of the different repeatability assays were less than 10% for the four tested levels. The mean percentages of error ranged from −8.1 to +10.9% for plasma concentrations varying between 2 and 40 ng ml^{-1} (Table I).

Standard curves

The seven-point calibration graphs obtained on each day of the assay, by plotting the peak-area ratios of *m/z* 505 (I), 449 (II), 393 (III) and 374 (I.S.) *versus* the known plasma or urine concentrations of the corresponding compound, were straight lines over the studied range of concentrations (1–40 ng ml^{-1}). The least-squares regression analysis passed near the origin, with intercept values close to zero.

TABLE I

PRECISION AND ACCURACY OF THE HPLC–MS METHOD

Compound	Added concentration (ng ml^{-1})	Measured concentration (mean ± S.D., n = 8) (ng ml^{-1})	C.V. (%)	Error (%)
BDP	2	1.84 ± 0.133	7.2	− 8.1
	5	5.07 ± 0.429	8.5	+ 1.4
	10	10.25 ± 1.016	9.9	+ 2.5
	40	36.90 ± 2.304	6.2	− 7.8
BMP	2	2.22 ± 0.159	7.2	+10.9
	5	4.94 ± 0.306	6.2	− 1.2
	10	9.56 ± 0.466	4.9	− 4.4
	40	40.73 ± 2.758	6.8	+ 1.8
BOH	2	2.01 ± 0.136	6.8	+ 0.4
	5	5.05 ± 0.367	7.3	+ 1.0
	10	10.18 ± 0.512	5.0	+ 1.8
	40	39.82 ± 1.413	3.5	− 0.5

Limit of detection

A previously published paper deals with the determination of a quantification limit based on the results obtained after the analysis of ten different blank plasma extracts [33]. During this LC–MS experiment, the single-ion monitoring traces of blank samples were free of endogenous interferences at the retention times of I, II and III. The limit of quantification was then fixed at 1 ng ml^{-1} for the three compounds. A representative mass chromatogram recorded after the LC–MS analysis of a blank plasma sample is shown in Fig. 3. Chromatograms from control specimens spiked with 1 and 40 ng ml^{-1} are presented in Figs. 4 and 5. When a plasma extract corresponding to the quantification limit of the method was analysed, the signal measured was equal to *ca.* 120 pg injected.

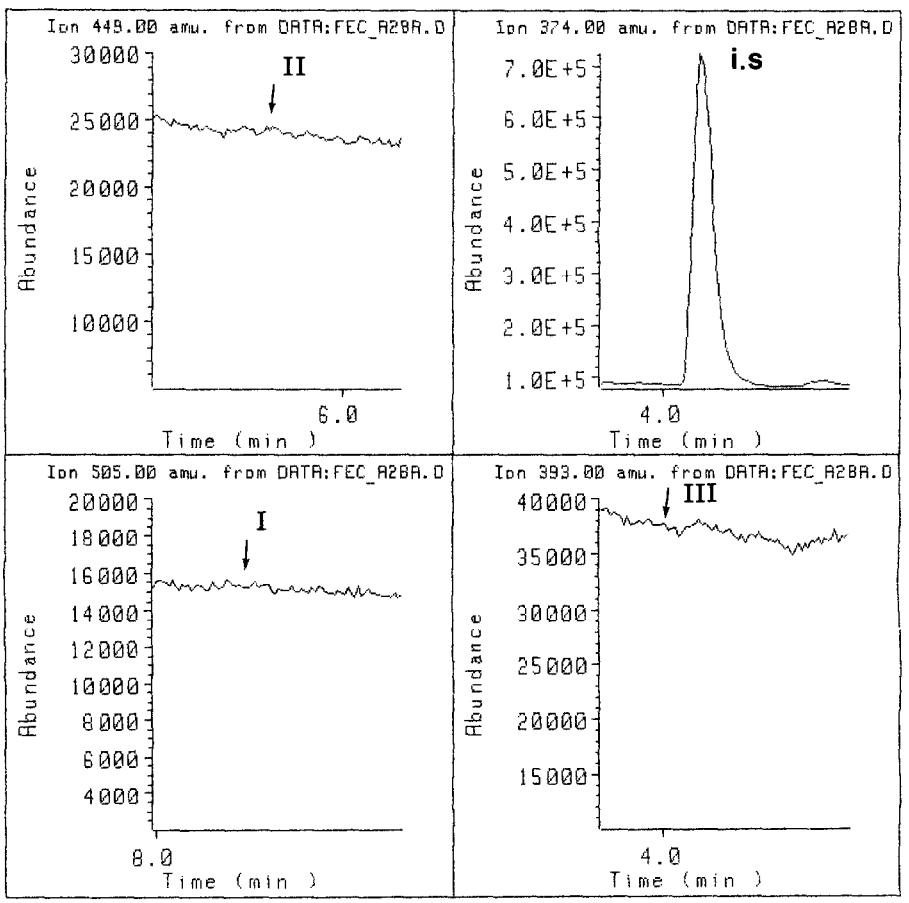

Fig. 3. Representative mass chromatogram recorded after the LC–MS analysis of a blank plasma sample (I.S. 25 ng ml^{-1}).

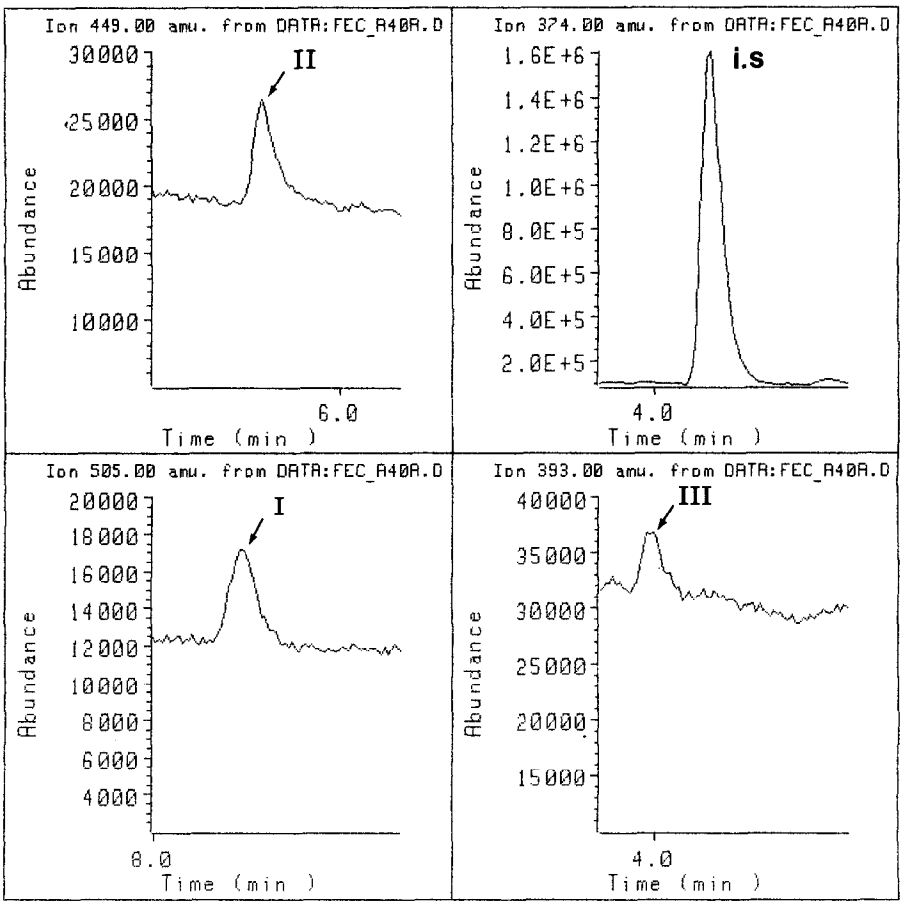

Fig. 4. Mass chromatogram obtained for a plasma sample spiked with 1 ng ml^{-1} of I, II and III (I.S. 25 ng ml^{-1}).

Pharmacokinetic data

Many patients with idiopathic inflammatory bowel disease who require long-term or intermittent therapy with oral or rectal formulations of systemically absorbed corticosteroids suffer from side-effects. Obviously, it would be preferable to treat these patients with a locally acting drug that could be effective in such low doses that a minimal amount of drug would be absorbed to produce systemic toxicity.

Beclomethasone 17,21-dipropionate may fulfill these requirements, and a study protocol was designed to evaluate the systemic absorption of the drug in the young healthy volunteer. The results have shown that the plasma concentrations of I, II and III were continuously lower than 1 ng ml^{-1}, thus indicating the

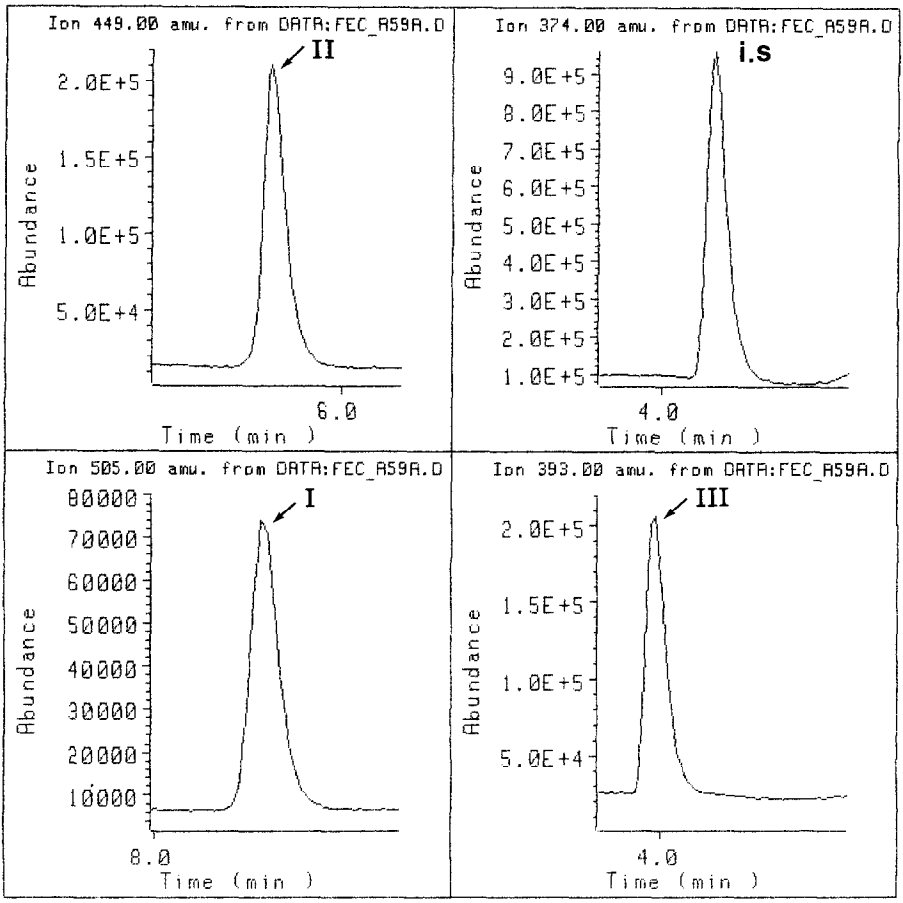

Fig. 5. Mass chromatogram obtained for a plasma sample spiked with 40 ng ml^{-1} of I, II and III (I.S. 25 ng ml^{-1}).

low systemic absorption of the drug. At this concentration no side-effects were observed.

CONCLUSION

The analysis of thermally labile compounds, such as corticosteroids, requires mild operating methods. The particle beam interface from Hewlett Packard was successfully used for the determination of beclomethasone 17,21-dipropionate and its hydrolysis products in human plasma and urine. When the characteristic high-mass ions (*m/z* 505, 449 and 393) are monitored under NICI conditions, the detection limit of this assay is 1 ng ml^{-1}. The values of the C.V. and mean percentage error calculated during the different repeatability assays demonstrate

that, even around the limit of detection, the precision and accuracy of the method are suitable for routine analysis of the three tested compounds. Moreover, the fast extraction procedure and the absence of a prolonged derivatization step allow the analysis of a great number of samples per day.

In conclusion, this specific and sensitive assay is suitable for pharmacokinetic and therapeutic drug monitoring of I, II and III. This method has been routinely used in our laboratory and could be extended to other synthetic corticosteroids.

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