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DETERMINATION OF ROXATIDINE IN HUMAN PLASMA, URINE AND MILK BY CAPILLARY GAS CHROMATOGRAPHY USING NITROGEN-SELECTIVE DETECTION

J.L. BURROWS*, K.W. JOLLEY and D.J. SULLIVAN

Hoechst Pharmaceutical Research Laboratories, Walton Manor, Walton, Milton Keynes MK7 7AJ (U.K.)

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SUMMARY

An assay is described for the determination of roxatidine in human plasma, urine and milk by gas chromatography. Roxatidine is extracted from the basified matrix with dichloromethane and esterified with propionic anhydride prior to analysis of the extracts by capillary gas chromatography using a nitrogen-specific detector. Detection limits are 5 ng/ml for plasma and milk and 1 μg/ml for urine, making the assay suitable for obtaining pharmacokinetic data from volunteer trials.

INTRODUCTION

Roxatidine acetate, 2-acetoxy-N-[3-[*m*-(1-piperidinylmethyl)phenoxy]-propyl] acetamide hydrochloride (I, Fig. 1) is a histamine H_2-receptor antagonist recently introduced for the treatment of gastric and duodenal ulcers. The parent compound is rapidly deacetylated and cannot be found in plasma samples taken from volunteers after oral administration. An assay was therefore required for the major circulating species (roxatidine, II, Fig. 1) to provide data from pharmacokinetic studies.

Assays for roxatidine in biological fluids from rat have been reported using both gas chromatography–mass spectrometry (GC–MS) [1] and high-performance liquid chromatography (HPLC) [2]. GC–MS is an unsuitable technique in most laboratories for the analysis of large numbers of samples and, as the UV absorption of roxatidine is poor except at low wavelength [2], detection of low concentrations is difficult by HPLC. An assay based upon capillary GC and nitrogen-selective detection was therefore developed which had the capability to analyse the large number of samples generated from pharmacokinetic studies in volunteers.

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EXPERIMENTAL

Reagents and solvents

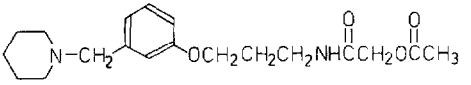
Roxatidine acetate (hydrochloride salt) and roxatidine (hemioxalate salt) were obtained from Teikoku Hormone (Tokyo, Japan). The internal standard (III, Fig. 1) was synthesised in the laboratories of Hoechst Pharmaceuticals (Milton Keynes, U.K.) and was supplied as the hemioxalate salt. Dichloromethane, dichlorodimethylsilane, ethyl acetate, hexane fraction, methanol, sodium hydroxide and toluene were purchased from Fisons (Loughborough, U.K.) and propionic anhydride was obtained from Aldrich (Gillingham, U.K.). Dichloromethane was routinely re-distilled before use.

Glassware

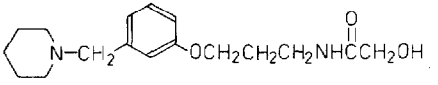
All test tubes used in the preparation of the extracts were treated with a 5% (v/v) solution of dichlorodimethylsilane in hexane (2 ml), rinsed with methanol (2 ml) and dried in an oven at 100°C before use.

Preparation of plasma extracts

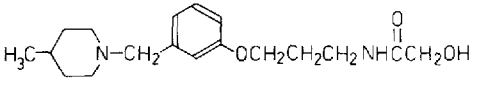
An aqueous solution of the internal standard (0.5 μg in 100 μl) was added to each 8-ml screw-cap glass test tube (J. Bibby, Stone, U.K.). The plasma sample (1 ml) was added and thoroughly mixed with the internal standard using a vortex mixer. Dichloromethane (4 ml) was added and the plasma made alkaline with 0.1 *M* sodium hydroxide (0.5 ml) before the samples were extracted for 5 min on a rotary inversion mixer (Heto Rotamix, V.A. Howe, London, U.K.) operating at 20 rpm. The phases were separated by centrifugation (2000 *g* for 5 min) and the plasma phase was aspirated. Dichloromethane often remained as an emulsion and this was broken by briefly placing the tubes on a vortex mixer. The tubes



I



II



III

Fig. 1. Structures of roxatidine acetate (I), roxatidine (II) and internal standard (III).

were then centrifuged again before the remaining plasma residues were aspirated. The dichloromethane was transferred to a tapered glass test tube (Quickfit, Fisons), placed in a heating block (Grant, Baird and Tatlock, Romford, U.K.) at 40–50°C and evaporated to dryness under a gentle stream of nitrogen. A freshly prepared 5% (v/v) solution of propionic anhydride in ethyl acetate (250 μl) was added to the residues which were dissolved with the aid of a vortex mixer. Esterification was completed by placing the tubes in the heating block at 80°C while the excess reagent was removed in a stream of nitrogen. Toluene (50 μl) was added and a vortex mixer was used to dissolve the residues. The extracts were transferred to 0.3-ml autosampler vials (Chromacol, London, U.K.) and 5-μl samples of these were analysed by GC.

Preparation of urine extracts

Urine samples were processed in the same way as plasma, but, because of the higher concentrations of roxatidine found in this matrix, the sample volume was reduced to 100 μl and the amount of internal standard added was increased to 10 μg. The volume of 0.1 *M* sodium hydroxide added to basify the sample was reduced to 0.1 ml and the volume of extract injected into the gas chromatograph reduced to 2 μl.

Preparation of milk samples

Milk samples (approximately 2 ml) were pipetted into 8-ml screw-cap glass test tubes and then centrifuged (2000 *g* for 5 min). The upper fatty layer was aspirated and discarded. A portion (1 ml) of the remaining milk was taken and processed in the same way as plasma samples.

Gas chromatography

The samples were analysed on a Model 5880A gas chromatograph (Hewlett-Packard, Winnersh, U.K.) equipped with a nitrogen-selective detector and split/splitless capillary inlet system operating in the split mode. Samples were injected with a Model 701 RN 10 μl syringe (Hamilton, V.A. Howe) attached to a Model 7672A autosampler using toluene as the wash solvent.

A fused-silica capillary column (25 m$\times$0.31 mm I.D.) coated with a 0.52-μm film of cross-linked methyl silicone (Part No. 19091A Opt. 112, Hewlett-Packard) was used for the analysis. A 2 mm I.D. silica liner partially packed with 3% OV-1 on Chromosorb W HP (100–120 mesh) was used in the injection port, as previously described [3]. The packing was replaced with fresh material after the analysis of each batch of samples and conditioned at the analysis temperature for several hours before use.

Helium was used as the carrier gas with an inlet pressure of 0.9 kg/cm^2 giving rise to a mean linear velocity of 28 cm/s. The split flow was 25 ml/min, detector make-up 25 ml/min and septum purge 1–2 ml/min. The flow-rates of the detector gases, hydrogen and air, were 3 and 60 ml/min respectively. The injection port and detector temperatures were 300°C and the oven was operated isothermally at 280°C. A baseline offset current of about 20 pA was set by adjusting the power to the detector bead.

Calibration procedure

The validation experiments showed the response to be linear and so quantification of unknown samples was based upon the peak-area ratios of roxatidine to the internal standard. Known amounts of roxatidine (0.5 μg/ml for plasma and milk, 100 μg/ml for urine) were added to the appropriate matrix and analysed with each batch of samples to determine the response factor. The day-to-day and within-day variations in the response factor were used as a check on assay performance. Blank samples plus quality control samples having known quantities of roxatidine covering the working ranges of the assay were also included and provided a daily check on the assay linearity.

RESULTS

Validation samples were prepared by adding aqueous solutions of roxatidine (hemioxalate salt) to blank matrix (plasma, urine or milk) to produce three series each containing nine to twelve samples with known concentrations over the required ranges of the assay. Each of these series was analysed on six separate days by the methods described. The results from three concentrations in each matrix are given in Tables I–III.

Chromatograms of plasma and urine extracts are shown in Figs. 2 and 3. These samples were analysed on the chromatographic system described except that the chromatograms were obtained via output to a 3350X data system (Hewlett-Packard).

TABLE I

RECOVERY OF ROXATIDINE AFTER ADDITION TO PLASMA

Roxatidine added (ng/ml)	Roxatidine found* (ng/ml)	Recovery* (%)	Coefficient of variation (%)
0	1.0 ± 1.4		
10.3	12.1 ± 2.0	117.5 ± 19.4	16.5
103	103 ± 4	100.0 ± 3.9	3.9
1028	1037 ± 31	100.9 ± 3.0	3.0

*Each result is the mean ± standard deviation of six results.

TABLE II

RECOVERY OF ROXATIDINE AFTER ADDITION TO URINE

Roxatidine added (μg/ml)	Roxatidine found* (μg/ml)	Recovery* (%)	Coefficient of variation (%)
0	0.28 ± 0.18		
5.00	4.82 ± 0.18	96.4 ± 3.6	3.7
50.1	50.0 ± 1.1	99.8 ± 2.2	2.2
506	546 ± 46	107.9 ± 9.1	8.4

*Each result is the mean ± standard deviation of six results.

TABLE III

RECOVERY OF ROXATIDINE AFTER ADDITION TO MILK

Roxatidine added (ng/ml)	Roxatidine found* (ng/ml)	Recovery* (%)	Coefficient of variation (%)
0	0 ±0		
26.4	25.4±1.2	96.2±4.5	4.7
260	253 ±12	97.3±4.6	4.7
2584	2611 ±84	101.0±3.3	3.2

*Each result is the mean ± standard deviation of six results.

Precision

Estimates of the precision of the assay were made from the six sets of results from the analysis of the series of validation samples from each matrix. Plasma and milk determinations showed similar precision and at low concentrations (5–50 ng/ml) the standard deviation lying in the ranges ± (0.5–3.3 ng/ml) and ± (1.4–4.7 ng/ml), respectively. As the concentration increased so did the estimates of the standard deviation and over the concentration range 0.25–2.5 μg/ml were found to be in the ranges ± (9–110 ng/ml) for plasma and ± (12–84 ng/ml) for milk. However, the coefficient of variation over this range is fairly constant with values of 3.0–3.9% (plasma) and 3.2–4.7% (milk).

The standard deviations of the urine measurements were ± (0.05–0.18 μg/ml) over the concentration range 0.5–5 μg/ml increasing to ± (1–46 μg/ml) over the 50–500 μg/ml concentration range. The within-day precision of the plasma assay at 500 ng/ml, as determined from the calibration samples, was 1.8±0.6% ($n=6$).

Limit of detection

If the detection limit (DL) is defined as the concentration at which the measured value is significantly greater than zero, then this may be expressed in terms of the limiting standard deviation as the concentration tends to zero ($SD_{c\to 0}$) by the equation $DL = t_{(n,95\%)} \cdot SD_{c\to 0}$. The value of $t_{(n,95\%)}$, which is the one-tailed critical value of the t-distribution for n determinations at the 95% confidence limit, is 2.0 for $n=6$. The mean values for precision at low concentrations yield a detection limit of about 5 ng/ml for plasma and milk. A detection limit of about 0.4 μg/ml is given for urine, but this was raised to 1 μg/ml for routine use due to the variable nature of the matrix and the high levels found.

Accuracy

The estimated concentrations of the validation samples were found to be within ±10% of the amount added in each matrix after correction for background. Endogenous material found in the pooled plasma used for the validation (Table I) was not usually present in actual samples and corrections for background were only made if levels exceeded the detection limit. The consistency of the day-to-day values of the response factor of roxatidine relative to the internal standard provides a check on the accuracy and was found to average 0.998 ±0.012 for the six plasma validations.

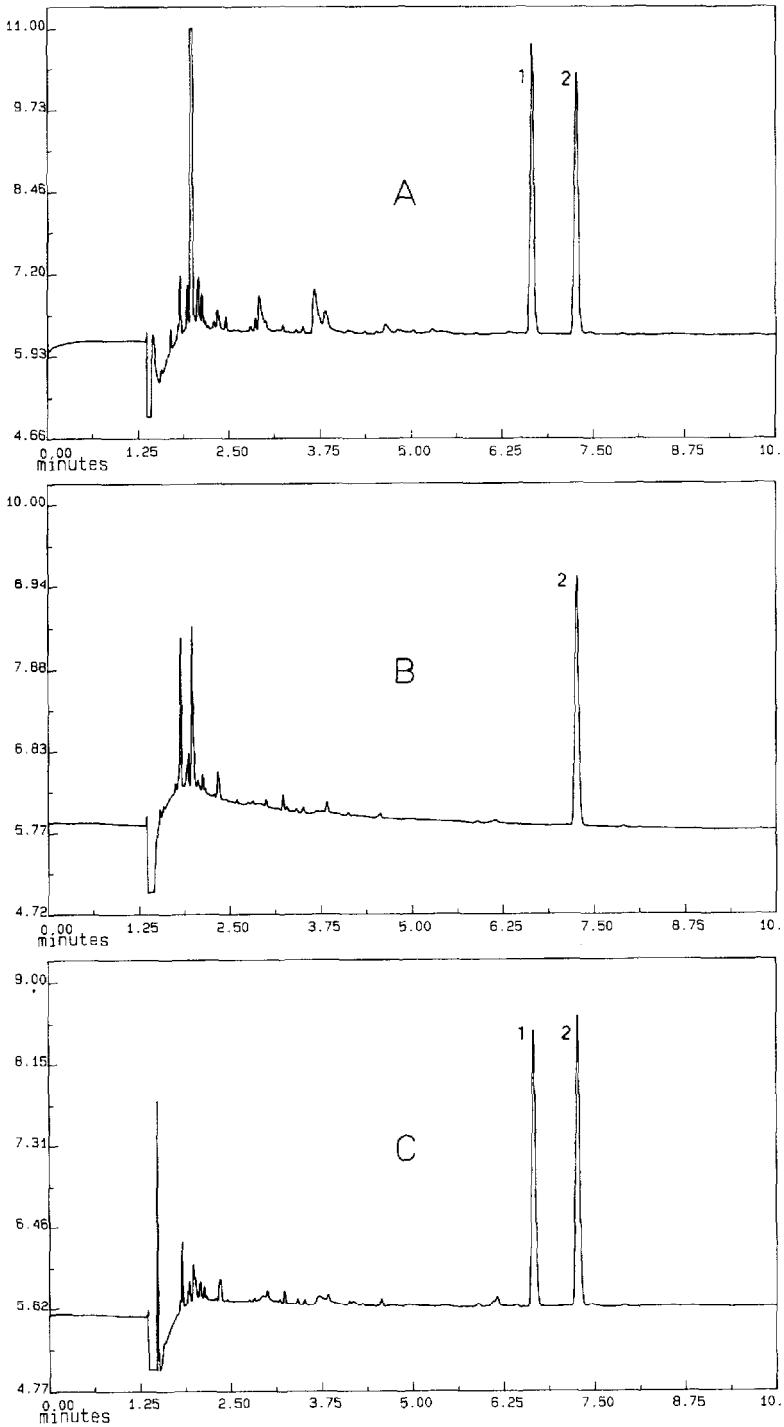


Fig. 2. Chromatogram of plasma extracts. (A) Calibration sample containing 0.5 μg/ml each of roxatidine (1) and internal standard (2); (B) pre-dose sample from a volunteer; (C) sample from a volunteer taken 4 h after administration of roxatidine acetate (150 mg).

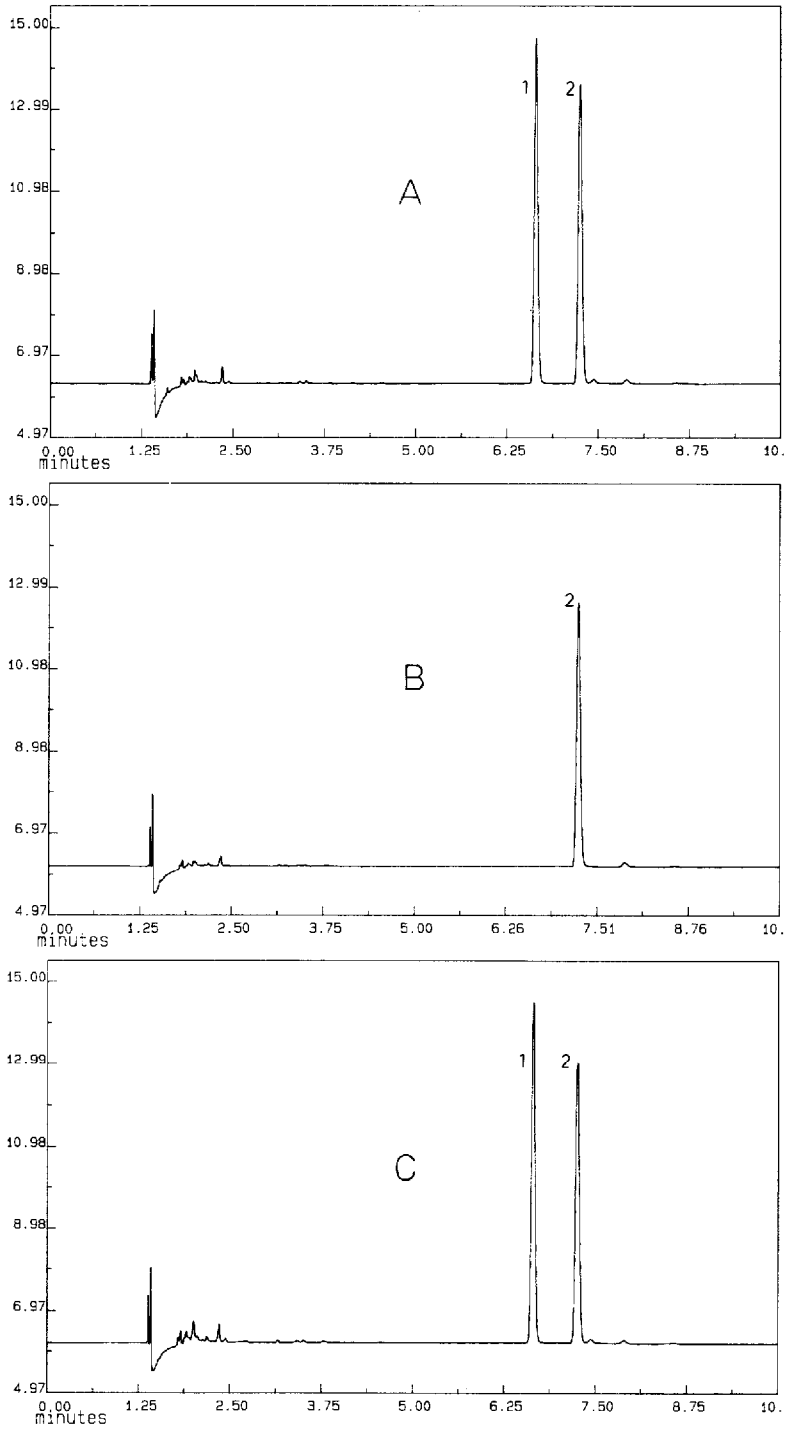


Fig. 3. Chromatograms of urine extracts. (A) Calibration sample containing 0.1 mg/ml roxatidine (1) and internal standard (2); (B) pre-dose sample from a volunteer; (C) 0–12 h urine sample from day 2 of a multiple-dose study (150 mg roxatidine acetate per day).

TABLE IV

RETENTION INDICES OF ROXATIDINE ANALOGUES ON METHYL SILICONE CAPILLARY AT 280 °C

Compound	Retention index
I	2743
II	2686
II O-propionate	2823
III	2737
III O-propionate	2871

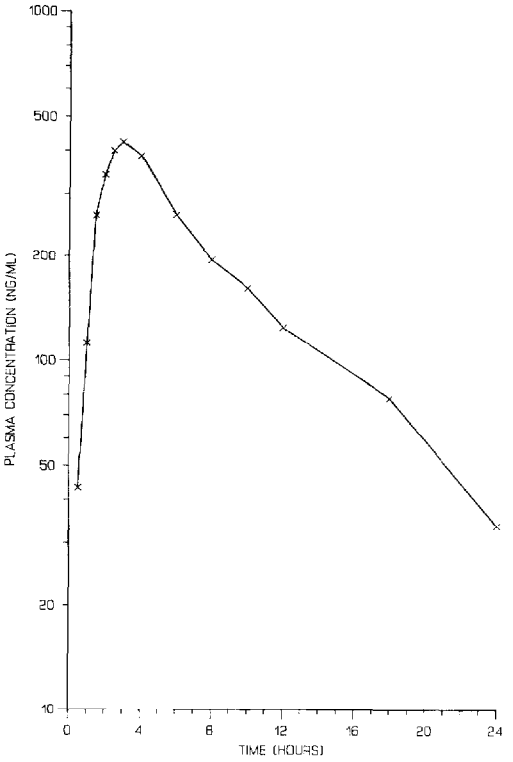


Fig. 4. Plasma levels of roxatidine after oral administration of roxatidine acetate (150 mg) to a volunteer.

Specificity

The assay was intended for use in healthy volunteer studies and so interference from co-administered drugs has not been a problem. Retention indices of the analytes are given in Table IV and can be used to assess possible interference from plasticisers or other drugs by comparison of retention data in published tables [4–7]. Roxatidine acetate is well separated from roxatidine by capillary GC and would not interfere with the assay if it were present in the samples.

DISCUSSION

Tailing peaks were produced when II and III are analysed by GC because the presence of free hydroxyl groups in these molecules results in column absorption.

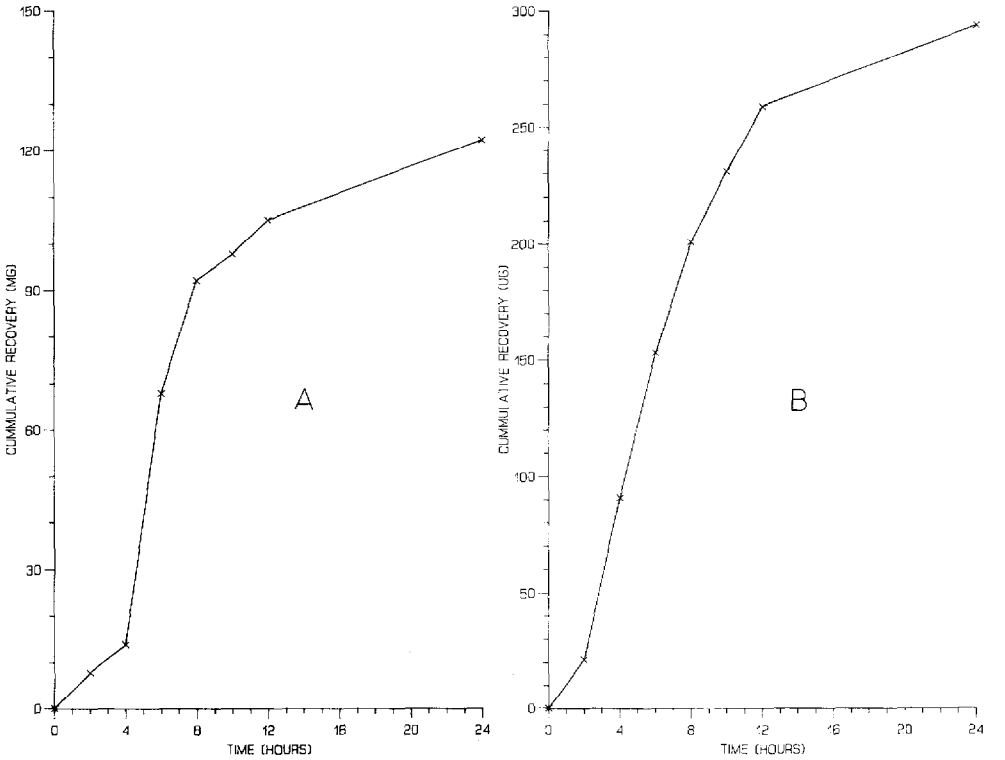


Fig. 5. Comparison of the recoveries of roxatidine in urine (A) and milk (B) after oral administration of roxatidine acetate (150 mg) to a lactating woman.

Quantification is therefore poorer because of a non-linear and non-reproducible response. It was therefore decided to form esters of II and III to improve their GC properties.

Trifluoroacetic anhydride is a reagent which is convenient to use because its volatility enables the excess reagent to be easily removed by evaporation, but was not used in this method due to the formation of side products. Formation of the acetates of II and III was not considered as the assay would no longer be able to demonstrate the absence of the parent compound in the samples.

Propionates of II and III were therefore used in the analysis despite the relatively high boiling point of propionic anhydride (167°C). Removal of the excess reagent under nitrogen did not present any practical difficulties nor lead to a concomitant loss of roxatidine or internal standard by evaporation.

The quantity of endogenous material in the extracts which enters the capillary column is limited by the use of a splitter in the injection port and batch sizes of up to 100 samples have been analysed without deterioration in column performance. Occasionally a build up of lipids from plasma or milk on the capillary column does occur which results in a loss of performance. This can be usually restored by maintaining the column under the operating conditions overnight and only rarely is it necessary to resort to rinsing the column or removing a few centimeters

from the inlet. Isothermal analysis also permits a faster throughput of samples and this factor, plus the robustness of the chromatography, compensates for the loss of any additional sensitivity which could be gained using splitless techniques.

Milk samples presented additional problems in that their high viscosity prevented an accurate volume being pipetted for analysis and the large amounts of fats in the extracts rapidly fouled the capillary column. Removal of the fats after centrifugation left a matrix suitable for analysis. Calibration and quality control samples were processed in the same way to compensate for any unequal partition of roxatidine during the separation and removal of fats.

Application

This assay has been used to determine roxatidine levels in over 10 000 samples from volunteer trials. In one such trial, roxatidine acetate (150 mg) was given to lactating women and samples of blood, urine and milk were collected. Plasma was obtained by centrifugation of the blood and all samples were stored deep frozen (−20°C) until analysed by the methods described. Part of a plasma profile from one of these volunteers is shown in Fig. 4 and the cumulative recoveries in urine and milk over the first 24 h are shown in Fig. 5. Maximum roxatidine concentrations in the milk and urine samples from this subject were 995 ng/ml and 135 μg/ml, respectively.

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