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ASSAY FOR N^{τ}-METHYLIMIDAZOLEACETIC ACID, A MAJOR METABOLITE OF HISTAMINE, IN URINE AND PLASMA USING CAPILLARY COLUMN GAS CHROMATOGRAPHY–NEGATIVE ION MASS SPECTROMETRY

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SUMMARY

A gas chromatographic–mass spectrometric assay has been developed for the measurement of N^{τ}-methylimidazoleacetic acid in urine and plasma. The method uses the isopropyl ester 3,5-bistrifluoromethylbenzoyl derivative of N^{τ}-methylimidazoleacetic acid and electron capture negative ion chemical ionisation mass spectrometry. The derivative has very good chromatographic properties and a negative ion mass spectrum which contains only a molecular ion at m/z 422. When this ion is specifically monitored, an amount of derivative equivalent to 1 pg of parent compound can be detected. A deuterated analogue of N^{τ}-methylimidazoleacetic acid was synthesised for use as an internal standard and this allowed the development of an assay for N^{τ}-methylimidazoleacetic acid, in urine with a precision of 2.9% and in plasma with a precision of 1.5%.

INTRODUCTION

Histamine (I, Fig. 1) is believed to be an important chemical mediator in hypersensitivity reactions such as hayfever and asthma [1]. Biochemical in-

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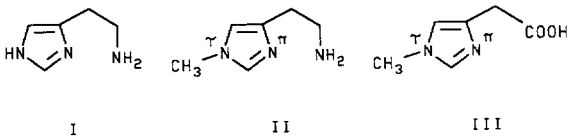

I II III

Fig. 1. Chemical structures of histamine (I) and its metabolites N^{τ}-methylhistamine (II) and N^{τ}-methylimidazoleacetic acid (III).

vestigation of these diseases therefore requires the development of methods for measuring histamine release before, during and after pathological episodes. Once released from storage granules in mast cells and basophils, histamine is rapidly metabolised in the blood stream with a half-life of only a few minutes [2]. Consequently, levels of the parent compound in blood and urine may not be accurate indicators of total histamine release. Attention has therefore focussed on the major products of histamine metabolism, N^{τ}-methylhistamine (II) and N^{τ}-methylimidazoleacetic acid (N^{τ}-MIAA, III) (Fig. 1). The latter is of especial interest as it accounts for approximately 60% of total histamine and is generally considered to be a unique metabolite of histamine. Several methods for the measurement of N^{τ}-MIAA in biological fluids have been reported in the literature. These include determination of urinary levels of the metabolite using gas chromatography (GC) [3–5] and GC–positive ion mass spectrometry (MS) [6–8]. The latter technique has also been used for the analysis of N^{τ}-MIAA in cerebrospinal fluid [7] and plasma [6,7,9]. We have developed and describe here a very specific and sensitive assay for N^{τ}-MIAA in urine and plasma using capillary column GC–negative ion MS.

EXPERIMENTAL

Chemicals

N^{τ}-MIAA, imidazole-4-acetic acid, [$^2H_6$]dimethyl sulphate, diisopropylethylamine and undecane were obtained from Sigma (Poole, U.K.). Stock solutions of N^{τ}-MIAA in 0.01 *M* HCl (1 μg/ml and 100 ng/ml) were prepared and stored at 4 °C until required. Bondelut® SCX disposable solid-phase extraction columns (1 ml capacity, Analytichem International) were supplied by Jones Chromatography (Hengoed, U.K.). Reactivials® (1 ml capacity) were purchased from Pierce and Warriner (Chester, U.K.) while propan-2-ol (HPLC grade) and acetyl chloride were purchased from Aldrich (Gillingham, U.K.). 3,5-Bistrifluoromethylbenzoyl (bis-TFMBO) chloride was obtained from Fluorochem (Glossop, U.K.). Methanol, hexane, ethyl acetate and concentrated HCl were all of Analar grade. Dry ethyl acetate was generated by distillation from and storage over calcium hydride.

Synthesis of N^{τ}-trideuteromethylimidazoleacetic acid (N^{τ}-[$^2H_3$]MIAA)

Keyzer et al. [5] have described the synthesis of N^{τ}-ethylimidazoleacetic acid from imidazole-4-acetic acid. The same reaction conditions were used for the preparation of N^{τ}-[$^2H_3$]MIAA except that [$^2H_6$]dimethyl sulphate was used in place of diethyl sulphate. The isopropyl ester of N^{τ}-[$^2H_3$]MIAA was characterised prior to hydrolysis by GC–electron impact MS: *m/z* 185 ($M^{+\cdot}$, 25), 126 (5), 98 (100), 81 (13). Stock solutions of N^{τ}-[$^2H_3$]MIAA in 0.01 *M* HCl (10 and 1 μg/ml) were prepared and stored at 4°C until required.

Preparation of samples for extraction

Urine. Urine (10 ml) was mixed with 2 ml of 1 *M* HCl. A 1.2 ml volume of the resultant solution was taken and diluted to 10 ml with deionised water. To a 1-ml aliquot of the diluted solution (containing 100 μl of urine in 0.02 *M* HCl and with a pH of 2.0) were added 1.5 μg of N^{τ}-[$^2H_3$]MIAA in 150 μl of 0.01 *M* HCl. This sample was then extracted as described below.

Plasma. To 2 ml plasma were added 3 ml of 0.1 *M* HCl and 150 ng of N^{τ}-[$^2H_3$]MIAA in 150 μl of 0.01 *M* HCl. This sample (pH 2.0) was then extracted as described below.

Extraction procedure

Each sample was applied to a Bondelut SCX column (1 ml) which had been preconditioned with methanol (1 ml), water (1 ml), 1% acetic acid in water (3 ml) and water (1 ml), respectively. The column was washed with 0.01 *M* HCl (2 ml), after which N^{τ}-MIAA and N^{τ}-[$^2H_3$]MIAA were eluted with 6 *M* HCl (1.5 ml) into a 20-ml flat-bottomed glass vial. The latter was placed in a water bath at 30°C and the vial contents were evaporated to dryness under a gentle stream of nitrogen. The residue was transferred to a 1-ml reactivial (2×750 μl methanol) and, after evaporation of the solvent under nitrogen, derivatised as described below.

Derivatisation procedure

To each reactivial were added propan-2-ol (100 μl) and acetyl chloride (20 μl). The vial was tightly capped and then placed in a heating block at a temperature of 70°C for 90 min. After cooling, the reagents were removed by evaporation under nitrogen. To the residue were added 0.1 *M* HCl (100 μl) and hexane (500 μl). The vial contents were vortex-mixed, centrifuged and the upper organic layer was discarded. This washing procedure with hexane was repeated, then 0.5 *M* sodium carbonate solution (100 μl) was added to the aqueous phase and the alkaline product extracted with ethyl acetate (2×600 μl). The combined organic extract in a half-dram glass vial was evaporated to dryness under nitrogen. Dry ethyl acetate (50 μl) containing bis-TFMBO chloride (0.5 μl) and diisopropylethylamine (0.5 μl) was added and the reaction mixture left at room temperature for 30 min; it was then evaporated to

dryness under nitrogen. The residue was reconstituted in undecane (500 μl for urine samples and standards, 50 μl for plasma samples and standards) and 2-μl aliquots were injected into the gas chromatograph–mass spectrometer.

Gas chromatography–mass spectrometry

A Finnigan MAT 4500 combined GC–quadrupole MS system (Finnigan MAT, San Jose, CA, U.S.A.) was used. The gas chromatograph was equipped with a 30 m$\times$0.25 mm I.D. DB5 J & W fused-silica capillary column which was routed through the separator oven (maintained at 290°C) directly into the mass spectrometer ion source. Helium was used as carrier gas at a head pressure of 138 bar (20 p.s.i.). The gas chromatograph was fitted with a Grob-type capillary injector operated in the splitless mode and maintained at a temperature of 270°C. The gas chromatograph oven temperature was held at 160°C for 1 min, then raised to 280°C at 20°C min^{-1}. Under these conditions, the retention times of the isopropyl ester bis-TFMBO derivatives of N^{τ}-MIAA and N^{τ}-[$^2H_3$]MIAA were 6 min and of N^{π}-MIAA and N^{π}-[$^2H_3$]MIAA 6.2 min. The mass spectrometer was operated in the negative ion chemical ionisation (NICI) mode with an electron energy of 100 eV. Ammonia gas was admitted to an indicated ion source pressure of 0.4 Torr and the indicated ion source temperature was maintained at 150°C. The mass spectrometer was tuned to monitor negative ions at *m/z* 422 and 425 and data acquisition and reduction were performed by an INCOS data system using IDOS 2 software.

Standard curves

Urine samples. Six standards in 1-ml reactivials were prepared from stock solutions of N^{τ}-MIAA (1 μg per ml of 0.01 *M* HCl) and N^{τ}-[$^2H_3$]MIAA (10 μg per ml of 0.01 *M* HCl). The standards all contained 1.5 μg N^{τ}-[$^2H_3$]MIAA as well as amounts of N^{τ}-MIAA in the 0–250 ng range. After evaporation to dryness under nitrogen, standards were taken through the derivatisation procedure described above.

Plasma samples. Six standards in 1-ml reactivials were prepared from stock solutions of N^{τ}-MIAA (100 ng per ml of 0.01 *M* HCl) and N^{τ}-[$^2H_3$]MIAA (1 μg per ml of 0.01 *M* HCl). The standards all contained 150 ng N^{τ}-[$^2H_3$]MIAA as well as amounts of N^{τ}-MIAA in the 0–25 ng range. After evaporation to dryness under nitrogen, standards were taken through the derivatisation procedure described above.

RESULTS AND DISCUSSION

A compound suitable for use as an internal standard in the assay for N^{τ}-MIAA was required. Stable isotope labelled analogues are normally the compounds of choice when carrying out quantitative analysis by GC–MS. Consequently, N^{τ}-MIAA containing three deuterium atoms in the N-methyl group

(N^{τ}-[$^2H_3$]MIAA) was prepared. The synthesis involved trideuteromethylation of imidazole-4-acetic acid, followed by esterification with isopropanol and finally hydrolysis with HCl. The reaction scheme is a modification of that used by Keyzer et al. [5] to prepare N^{τ}-ethylimidazoleacetic acid, and these authors state that their final reaction product was a mixture of the N^{τ} and N^{π} isomers of N-ethylimidazoleacetic acid. When our final reaction product was derivatised and analysed by GC–MS as described below, it also was found to be a mixture of N^{τ}-[$^2H_3$]MIAA and N^{π}-[$^2H_3$]MIAA. Having an isomeric mixture for use as an internal standard is quite acceptable provided the two compounds are not readily interconvertible and are resolved by GC, and so no further purification was attempted.

For the development of an assay based on GC–MS, it is important to use derivatives of the compound of interest and the internal standard which have good GC properties and allow sensitive and specific detection by selected ion monitoring (SIM). In this laboratory, extensive use has been made of fluorobenzyl ester derivatives and electron capture NICI (ECNICI) MS for prostaglandin analysis [10,11]. Fluorobenzyl esters are readily formed by reaction of the carboxyl group in the prostaglandin molecule with a fluorobenzyl bromide. N^{τ}-MIAA also possesses this functional group and so the 3,5-bistrifluoromethylbenzyl ester of N^{τ}-MIAA was synthesised using a procedure described elsewhere [12] and its ECNICI mass spectrum recorded. The latter (Fig. 2) was found not to contain an abundant high-mass ion suitable for SIM work, the base peak in the mass spectrum at *m/z* 139 corresponding to the carboxylate

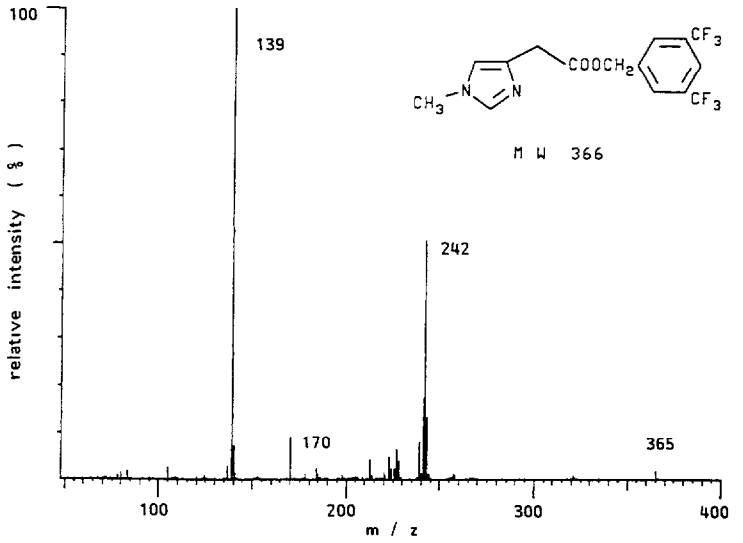

Fig. 2. Chemical structure and ECNICI mass spectrum of the 3,5-bistrifluoromethylbenzyl ester of N^{τ}-MIAA.

anion of the parent compound. Chromatographic behaviour was also very poor (a problem found previously with ester derivatives of N^{τ}-MIAA [5]) and so other derivatisation procedures were examined.

We have previously reported the use of bis-TFMBO derivatives and EC-NICI-MS in drug [13,14], drug metabolite [15] and steroid [16] analysis. This methodology has also been successfully applied in the development of an assay for the heterocyclic amine 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) in cooked meat and fish [17] as well as in the analysis of various biogenic amines [18]. The ECNICI mass spectra of the bis-TFMBO derivatives of most compounds previously studied contained molecular ions which were the base peaks in the mass spectra and there were few, if any, major fragment ions. SIM of these molecular ions, besides ensuring high specificity, also gave very good sensitivity since often an amount of derivative equivalent to as little as 1 pg of the parent compound could be detected.

The possibility of making a bis-TFMBO derivative of N^{τ}-MIAA was therefore examined. Other workers have synthesised carboxylic acid ester heptafluorobutyryl derivatives of N^{τ}-MIAA [7,8] and have found that these imidazole ring-substituted compounds show much better chromatographic behaviour than those where only the carboxylic acid side-chain has been modified [5]. The double derivatives were made by a two-step reaction scheme, the acyl group being inserted into the imidazole ring after the carboxylic acid side-chain had been esterified. Consequently the isopropyl ester bis-TFMBO derivative of N^{τ}-MIAA was synthesised by an analogous route and its ECNICI mass spectrum recorded (Fig. 3a). A molecular ion at *m/z* 422 was the base peak in the mass spectrum and there were no major fragment ions. The derivative also had very good GC properties with no sign of peak broadening or tailing and, when the molecular ion was specifically monitored, an amount of derivative equivalent to 1 pg of N^{τ}-MIAA could be detected (Fig. 4). This compound, therefore, appeared to be a good choice for use in the analysis of N^{τ}-MIAA.

The isopropyl ester bis-TFMBO derivative of N^{τ}-[$^2H_3$]MIAA was then prepared. Because the synthesis of the deuterium-labelled imidazole had also produced isomeric N^{π}-[$^2H_3$]MIAA, when the derivatisation reaction product was analysed by GC–MS, two compounds were identified with differing GC retention times but identical mass spectra (a single major ion at *m/z* 425, Fig. 3b). The earlier eluting, major component was identified as the isopropyl ester bis-TFMBO derivative of N^{τ}-[$^2H_3$]MIAA since its retention time was virtually identical to that of the corresponding derivative of the unlabelled compound. The later eluting, minor component was therefore the double derivative of N^{π}-[$^2H_3$]MIAA.

Standards for urine analysis containing N^{τ}-MIAA (0–250 ng) and N^{τ}-[$^2H_3$]MIAA (1.5 μg) and standards for plasma analysis consisting of N^{τ}-MIAA (0–25 ng) and N^{τ}-[$^2H_3$]MIAA (150 ng), all in small volumes of 0.01 *M* HCl, were prepared. These solutions were evaporated to dryness, derivatised and

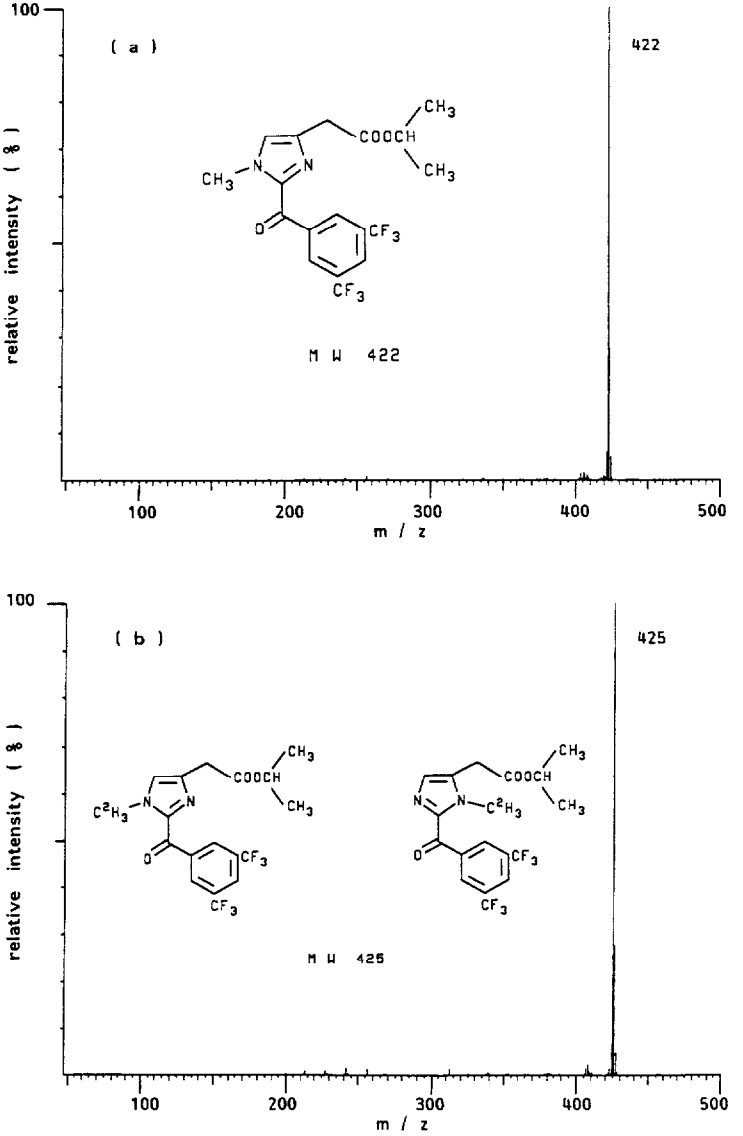

Fig. 3. Chemical structures and ECNICI mass spectra of (a) the isopropyl ester bis-TFMBO derivative of N^{τ}-MIAA and (b) the isopropyl ester bis-TFMBO derivatives of N^{τ}-[$^2H_3$]MIAA and N^{π}-[$^2H_3$]MIAA.

then analysed by GC–MS (a typical SIM trace is shown in Fig. 5). Over both ranges, the unextracted standard curves for N^{τ}-MIAA were linear with intercepts close to zero (Table I).

A procedure for the extraction of N^{τ}-MIAA from urine and plasma based on cation-exchange chromatography was used [5,8,9]. N^{τ}-MIAA possesses two

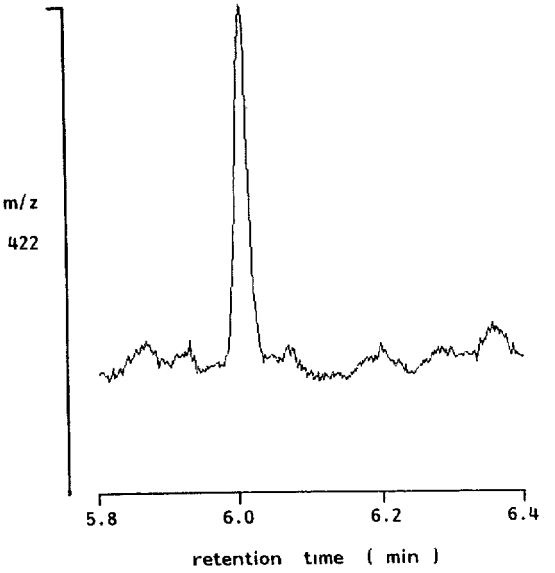

Fig. 4. SIM trace for the analysis of the isopropyl ester bis-TFMBO derivative of N^{τ}-MIAA (derivative equivalent to 1 pg of N^{τ}-MIAA injected on-column).

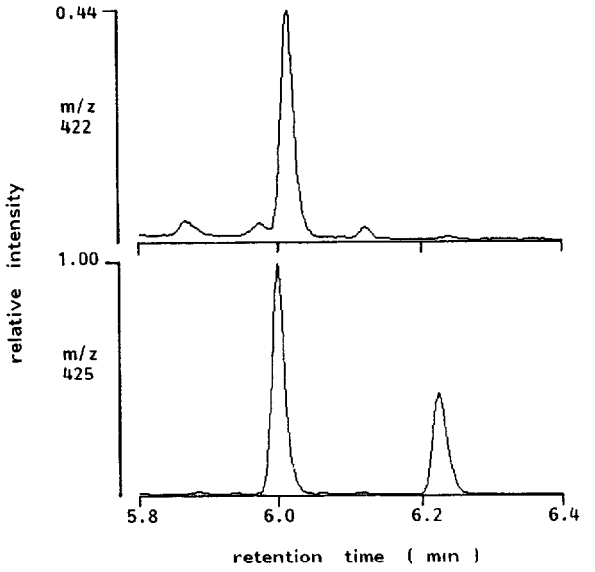

Fig. 5. SIM trace for the analysis of N^{τ}-MIAA in an unextracted standard.

ionisable groups, a carboxyl group with a $pK_a \sim 4$ and an imidazole ring with a $pK_a \sim 7$. Hence at pH 2, 99% of N^{τ}-MIAA present in a sample will carry a nett positive charge and can be isolated from the biological matrix by use of a strong

TABLE I

STANDARD CURVE PARAMETERS FOR THE MEASUREMENT OF N^{τ}-MIAA IN URINE AND PLASMA

Sample	Slope	*y*-Intercept	*r*
Urine (0–250 ng N^{τ}-MIAA)			
Unextracted	0.00416	0.006	1.000
Extracted from water	0.00417	0.012	1.000
Extracted from urine	0.00419	1.152	0.985
Plasma (0–25 ng N^{τ}-MIAA)			
Unextracted	0.0410	0.003	1.000
Extracted from water	0.0412	0.008	0.999
Extracted from plasma	0.0405	1.052	0.982

cation-exchange resin. A further purification step was incorporated into the derivatisation procedure where, after esterification with propan-2-ol–acetyl chloride, the sample was acidified and washed twice with hexane prior to basification and extraction with ethyl acetate. Using the extraction and derivatisation procedures described above, analysis by GC–MS of urine and plasma samples gave SIM traces (Fig. 6a and b, respectively) that were free of any interference and recovery through the extraction, assessed by comparison of internal standard peak areas in extracted samples with those in unextracted standards, was ~30%. Examination of the SIM traces for urine and plasma samples also showed the presence of a peak in the *m/z* 422 ion channel which corresponded to the isopropyl ester bis-TFMBO derivative of N^{π}-MIAA. N^{π}-MIAA has previously been detected in human urine [3,5] and it has been suggested that the source of this isomer of the histamine metabolite is in the diet [3].

Standards identical to those used for preparation of the unextracted standard curves were extracted from water, a urine sample and a plasma sample. The slopes of the extracted standard curves were the same as those of unextracted standard curves (Table I), indicating that N^{τ}-MIAA and N^{τ}-[$^2H_3$]MIAA had the same recovery through the extraction procedure. However, *y*-intercepts of the extracted standard curves from urine and plasma were displaced (Table I) by values corresponding to the amount of N^{τ}-MIAA already present in the biological fluid. The levels of N^{τ}-MIAA found in the urine sample (2.7 μg/ml) and the plasma sample (13.0 ng/ml) were within the normal ranges reported by other investigators [3,5,9]. Because the gradients of unextracted and extracted standard curves were found to be the same, unextracted standard curves were used for the routine analysis of urine and plasma samples. The precision for measurement of N^{τ}-MIAA in urine was ±2.9% (S.D., $n=6$) and for the analysis of N^{τ}-MIAA in plasma ±1.5% (S.D., $n=6$).

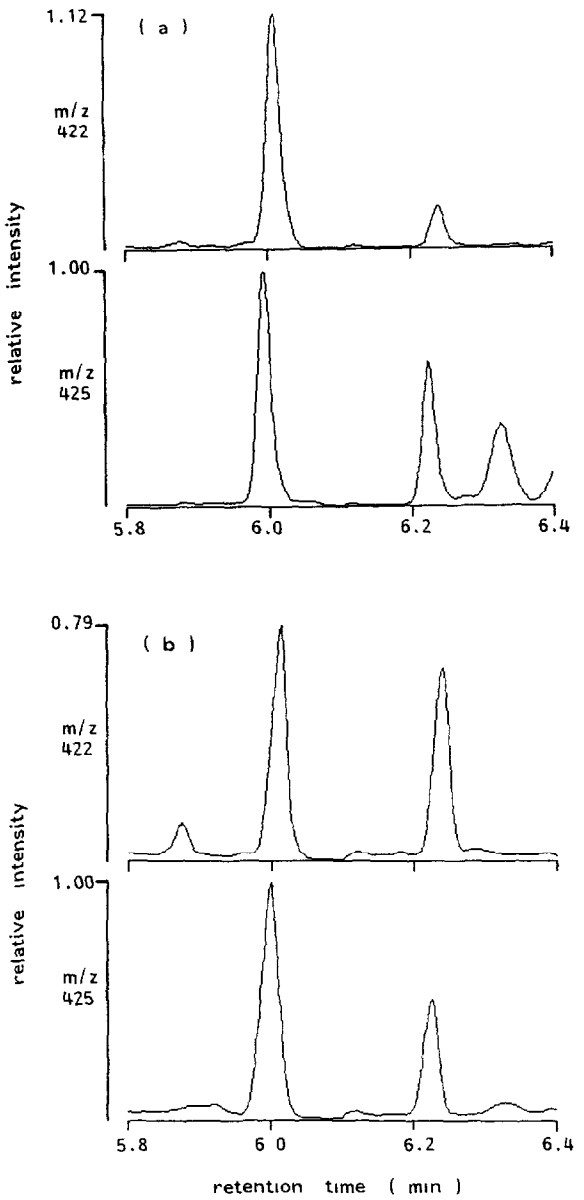

Fig. 6. SIM traces for the analysis of N^{τ}-MIAA in (a) urine and (b) plasma.

When histamine and N^{τ}-methylhistamine are treated with bis-TFMBO chloride as described here, di(bis-TFMBO) derivatives are formed. In both cases, the derivatives have good GC properties and their ECNICI mass spectra contain molecular ions (*m/z* 591 and 605, respectively) which are the base

peaks with few fragment ions. A di(bis-TFMBO) derivative would therefore appear to be a good choice as the basis of a GC–MS assay for histamine and N^{τ}-methylhistamine. Deuterated analogues of the two compounds are commercially available for use as internal standards and, because it would be of interest to us to measure histamine and N^{τ}-methylhistamine as well as N^{τ}-MIAA in urine and plasma, we are currently developing this methodology using this derivative.

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