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Gas chromatographic–mass spectrometric assay to measure urinary N^{τ}-methylhistamine excretion in man

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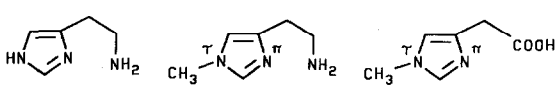
ABSTRACT

An assay has been developed for N^{τ}-methylhistamine, a major metabolite of the autocoid histamine, based on gas chromatography–electron-capture negative-ion chemical ionisation mass spectrometry. N^{τ}-Methylhistamine was extracted from urine by cation-exchange chromatography and converted to its di-(3,5-bistrifluoromethylbenzoyl) derivative. The latter has good chromatographic properties and gives a negative-ion mass spectrum with the molecular ion ($M^{-\cdot}$, *m/z* 605) as base peak. A commercially available trideuterated analogue of N^{τ}-methylhistamine was used as internal standard. Basal urinary excretion of N^{τ}-methylhistamine in five normal subjects was found to be 0.21 ± 0.05 μmol/h (289 ± 74 μmol/mol of creatinine). This value was not significantly altered in these subjects following the infusion of a sub-pharmacological dose of histamine. In eight atopic volunteers, basal urinary excretion of N^{τ}-methylhistamine was also not significantly changed following challenge with inhaled allergen.

INTRODUCTION

Histamine (I, Fig. 1) is a mast cell-derived product which has been implicated as an inflammatory mediator in diseases such as bronchial asthma and hay fever [1,2] and, in order to quantify mast cell activation, an index of histamine production would be of value. Measurement of histamine in plasma is complicated by the low basal levels (<0.4 ng/ml) and *ex vivo* production from basophils during venous sampling [3,4]. Measurement of histamine in urine is also an unreliable guide in that levels of the compound can be elevated due to the presence of endogenous bacteria and flora in the urogenital tract, expecially of females [5,6]. A major pathway of histamine metabolism in man is by N-methylation to N^{τ}-



I II III

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

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methylhistamine (N^{τ}-MH, II, Fig. 1) followed by oxidation to N^{τ}-methylimidazoleacetic acid (N^{τ}-MIAA, III, Fig. 1) [7]. We have previously demonstrated that there is a high basal urinary excretion of N^{τ}-MIAA and so this compound is not normally a suitable marker of endogenous histamine production in man [8]. In contrast, it has been suggested that urinary excretion of N^{τ}-MH more accurately reflects total body histamine production [9] and therefore, in order to explore this possibility further, an assay for N^{τ}-MH in urine was required.

Published methods for the measurement of N^{τ}-MH in urine make use of high-performance liquid chromatography with fluorescence [10,11] and electrochemical [12] detection, gas chromatography with electron-capture [13] and nitrogen–phosphorus [14,15] detection, and gas chromatography–mass spectrometry (GC–MS) with selected-ion monitoring (SIM) of positive ions [16–20]. Of the three analytical techniques, GC–MS provides the greatest specificity because of the high resolving power of modern capillary columns together with the selectivity of only recording certain prominent ions in the mass spectrum of the compound of interest. Specificity and sensitivity can be improved further, however, by using halogenated derivatives and by operating the mass spectrometer in the electron-capture negative-ion chemical ionisation (ECNICI) mode, and we have reported the use of bistrifluoromethylbenzoyl (bis-TFMBO) derivatives and ECNICI mass spectrometry as the basis of assays for a number of endogenous and exogenous compounds [21–23]. The negative-ion mass spectra of these derivatives usually contain molecular ions which are the base peaks in the mass spectra, with few, if any, major fragment ions. SIM of these molecular ions, besides being a highly specific method of detection, also offers very good sensitivity with detection limits of 1 pg readily achieved. Recently we have described the development of an assay for the histamine metabolite N^{τ}-MIAA in both urine and plasma using this methodology [24] and we therefore used a similar approach to develop a specific and sensitive assay for N^{τ}-MH. The latter was then used to measure basal production of this histamine metabolite in man, to assess the pharmacodynamics of N^{τ}-MH in man following infusion of histamine and to determine its urinary excretion in eight atopic subjects after challenge with inhaled allergen.

EXPERIMENTAL

Chemicals

N^{τ}-MH dihydrochloride, diisopropylethylamine and dodecane were obtained from Sigma (Poole, U.K.) while N^{τ}-[$^2H_3$]MH dihydrochloride was obtained from MSD Isotopes (Montreal, Canada). Bond Elut® CBA disposable solid-phase extraction columns (functional group, carboxymethyl, a weak cation exchanger; 1 ml capacity; Analytichem International) were supplied by Jones Chromatography (Hengoed, U.K.). 3,5-Bistrifluoromethylbenzoyl chloride (bis-TFMBO chloride) was purchased from Fluorochem (Glossop, U.K.). Histamine for infusion was supplied as a solution of its acid phosphate salt by MacArthys Pharmaceu-

tical (London, U.K.). Methanol, ethyl acetate and hydrochloric acid were all of Analar grade and dry ethyl acetate was generated by distillation from, and storage over, calcium hydride.

Extraction procedure

Deionised water was treated with a small volume of ammonia to adjust the pH to ~8. An aliquot of the ammoniacal deionised water (1.8 ml) and N^{τ}-[$^2H_3$]MH (100 ng free base in 100 μl deionised water) was added to urine (0.2–0.4 ml). This sample was then applied to a Bond Elut CBA column (1 ml) which had been preconditioned with 0.01 *M* hydrochloric acid (1 ml) and deionised water (3 ml), respectively. The column was washed with deionised water (1 ml), after which N^{τ}-MH and N^{τ}-[$^2H_3$]MH were eluted with 0.1 *M* hydrochloric acid (2 ml) into a 20 ml flat-bottomed glass vial. The latter was placed in a waterbath at 30°C and the vial contents were evaporated to dryness under a gentle stream of nitrogen. The residue was transferred to a half-dram glass vial (2 × 0.75 ml methanol) and, after evaporation of the solvent under nitrogen, derivatised as described below.

Derivatisation procedure

Dry ethyl acetate (100 μl) containing bis-TFMBO chloride (1 μl) and diisopropylethylamine (1 μl) was added and the reaction mixture left at room temperature for 1 h. The reagents were then removed by evaporation under nitrogen and to the residue was added saturated sodium bicarbonate solution (200 μl). The alkaline product was extracted with ethyl acetate (2 × 600 μl) and the combined organic extract in a clean half-dram vial evaporated to dryness under nitrogen. The residue was reconstituted in dodecane (20 μl for urine samples and 50 μl for 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 × 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 0.14 MPa. 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 200°C for 1 min, then raised to 320°C at 20°C/min. Under these conditions, the retention times of the major di(bis-TFMBO) derivatives of N^{τ}-MH and N^{τ}-[$^2H_3$]MH were 6.4 min. The mass spectrometer was operated in the ECNICI 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* 605 and 608, and data

TABLE I

GC AND MS PARAMETERS OF BIS-TFMBO DERIVATIVES OF N^{τ}-MH AND N^{τ}-[$^2H_3$]MH

Parent compound	Bis-TFMBO derivatives formed	Retention time (min)	MW of derivatives	*m/z* values (relative intensity) of major ions in negative-ion mass spectrum	Relative intensity[a] of molecular ions
N^{τ}-MH	Di	4.7	605	605 (100), 586 (4), 497 (13), 434 (11)	0.22
	Di	6.4	605	605 (100), 586 (3), 269 (4)	1.00
	Tri	6.5	845	845 (100), 497 (7), 348 (3)	0.17
N^{τ}-[$^2H_3$]MH	Di	4.7	608	608 (100), 589 (5), 497 (13)	0.39
	Di	6.4	608	608 (100), 589 (3), 269 (9), 213 (3)	1.00
	Tri	6.5	848	848 (100), 497 (8), 446 (11)	0.22

[a] Most abundant derivative in mixture = 1.

acquisition and reduction were performed by an INCOS data system using IDOS 2 software.

Standard curve

Six standards in half-dram glass vials were prepared from stock solutions of N^{τ}-MH and N^{τ}-[$^2H_3$]MH (1 μg free base per ml methanol). The standards all contained 100 ng N^{τ}-[$^2H_3$]MH as well as amounts of N^{τ}-MH in the 0–100 ng range. After evaporation to dryness under nitrogen, standards were taken through the derivatisation procedure described above.

Clinical studies

All studies were approved by the Ethics Committee of the Royal Postgraduate Medical School.

Histamine infusion. Five normal caucasian males aged 33–41 years were infused at the rate of 25 ml/h with normal saline for 1 h, followed by a 2-h infusion of either histamine acid phosphate at 50 ng free base/kg/min in saline or vehicle alone, in random order. The infusions were separated in time by at least two weeks. Heart rate, blood pressure and skin temperature were monitored throughout. The subjects voided their bladders immediately prior to the start of the histamine/saline infusion (0 h) and urine collections were made at time intervals of 1, 2, 3, 4, 6 and 8 h. Urine volumes were measured and fluid replacement was made with water up to and including the 3-h collection.

Allergen challenge. Eight atopic subjects (five female, three male, aged 19–41 years, mean age 28 years) inhaled increasing amounts of allergen (either *Dermatophagoides pteronyssius* or grass pollen extract) from a compressed air-driven nebuliser attached to a breath-activated dosimeter until a fall of $>20\%$ in FEV_1 (forced expiratory volume in 1 s) had been achieved. Urine was collected for 4 h post challenge. A 24-h control urine for the eight individuals was collected not less than two weeks later under the same experimental conditions.

Data analysis

Absolute values from the infusion study were compared by analysis of variance. Measurements from the allergen challenge study were analysed by Wilcoxon sign-rank analysis for within group-paired data. Values are expressed as mean $\pm$ S.E.M., and significance was assigned at $p < 0.05$.

RESULTS AND DISCUSSION

When treated with bis-TFMBO chloride at room temperature, N^{τ}-MH gave three reaction products (Table I). The major product was a di(bis-TFMBO) derivative, with a retention time of 6.4 min, and there were smaller amounts of another di(bis-TFMBO) derivative and a tri(bis-TFMBO) derivative. The amino group in N^{τ}-MH will react readily with bis-TFMBO chloride and therefore the

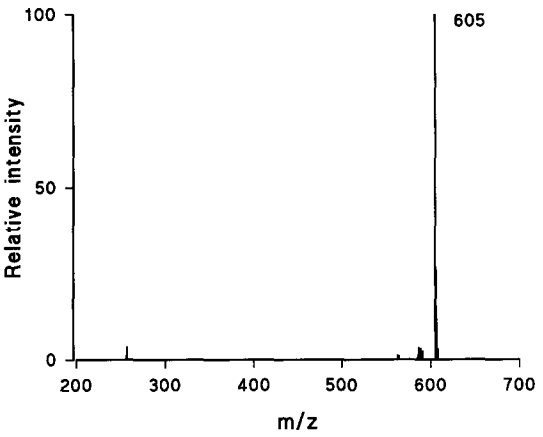

Fig. 2. ECNICI mass spectrum of the major di(bis-TFMBO) derivative of N^{τ}-MH.

formation of two di(bis-TFMBO) derivatives is due to substitution of a benzoyl group at one of two different positions in the imidazole ring. Disubstitution of the imidazole ring is unlikely to occur [24] and therefore the tri(bis-TFMBO) derivative also detected is probably formed by further reaction of the amide group in the sidechain of one of the di(bis-TFMBO) derivatives, a reaction that has been reported previously with amides and the reagent pentafluorobenzoyl chloride [25]. All three reaction products had good GC properties and every negative-ion mass spectrum showed an intense molecular ion with little fragmentation (Table I, Fig. 2). Although there was multiple product formation, the detection limit for N^{τ}-MH was good. When the molecular ion of the major product (*m/z* 605) was specifically monitored, an amount of derivative equivalent to 1 pg of N^{τ}-MH could be detected with a signal-to-noise ratio of >50.

The trideuterated analogue of N^{τ}-MH available for use as an internal standard (N^{τ}-[$^2H_3$]MH) gave the same mixture of derivatives when treated with bis-TFMBO chloride as N^{τ}-MH itself. The major product was a di(bis-TFMBO) derivative with a retention time of 6.4 min, and its negative-ion mass spectrum contained an intense molecular ion at *m/z* 608 as base peak (Table I). Standards containing N^{τ}-MH (0–100 ng) and N^{τ}-[$^2H_3$]MH (100 ng) were prepared, derivatised and then analysed by GC–MS. When monitoring the molecular ions of the major di(bis-TFMBO) derivatives of N^{τ}-MH and N^{τ}-[$^2H_3$]MH, the unextracted standard curve obtained was linear with an intercept close to zero (Table II).

Cation-exchange chromatography was used for the extraction of N^{τ}-MH from urine. N^{τ}-MH possesses two ionisable groups, an imidazole ring with a pK_a of ~ 7 and an amino group with a pK_a of ~ 10. By adjusting the pH of the urine to 8 with ammonia, N^{τ}-MH present in the sample is positively charged and can be isolated from the biological matrix with a weak cation-exchange sorbent (pK_a 4.8). Bound N^{τ}-MH is subsequently displaced from the sorbent with strong acid.

TABLE II

STANDARD CURVE PARAMETERS FOR THE MEASUREMENT OF N^{τ}-MH IN URINE

Standard curve[a]	Slope (m)	y-Intercept (c)	Correlation coefficient (r)
Unextracted	0.0159	−0.008	0.999
Extracted	0.0165	0.392	0.998

[a] $y = mx + c$, where y is the measured peak-area ratio of N^{τ}-MH to internal standard and x is amount (ng) of N^{τ}-MH present in the sample.

Using the extraction and derivatisation procedures described above, analysis by GC–MS of urine samples gave SIM traces (Fig. 3) 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 ~20%. Standards identical to those used for preparation of the unextracted standard curve were extracted from urine and analysed by GC–MS. The equation of the extracted standard curve (Table II) had the same slope as the unextracted standard curve but the y-intercept was substantially higher due to the endogenous N^{τ}-MH in the urine. The fact that the slopes of the extracted and

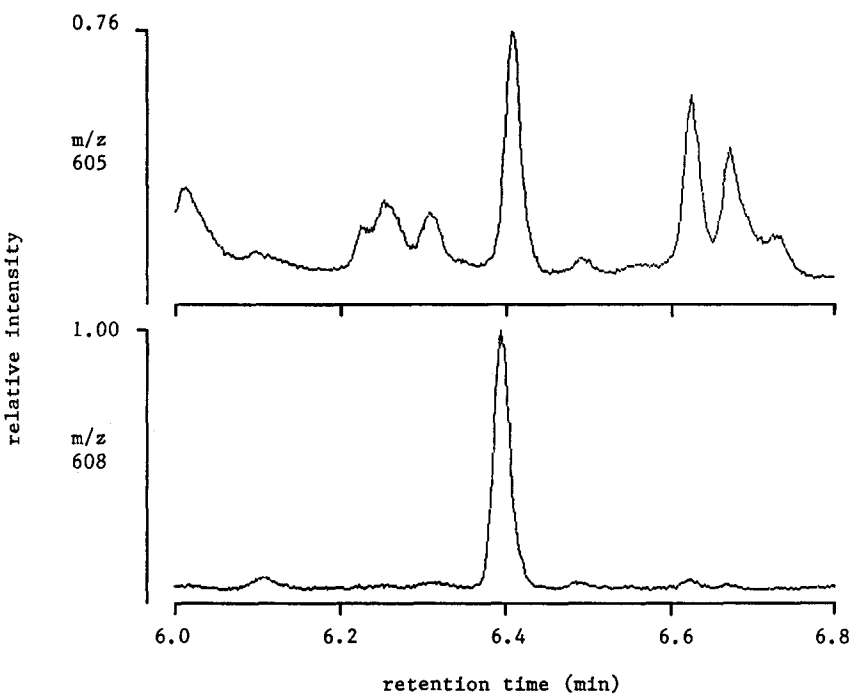

Fig. 3. SIM trace for the analysis of N^{τ}-MH in urine.

unextracted standard curves were the same indicated that N^{τ}-MH and N^{τ}-[$^2H_3$]MH had the same recovery through the extraction procedure, and so unextracted standard curves were used for the routine analysis of urine samples. The precision for measurement of N^{τ}-MH in urine was ±3.2% (S.D., n = 6).

The specific and sensitive assay for N^{τ}-MH described above was then used to investigate whether or not infusion of sub-pharmacological doses of histamine into normal human volunteers led to an increase in urinary excretion of N^{τ}-MH. Basal excretion in five subjects was determined as 0.21 ± 0.05 μmol/h (mean ± S.E.M.), equivalent to 289 ± 74 μmol/mol of creatinine, a value somewhat higher than that obtained by Oosting and co-workers [9,20,26]. This variation could well be due to differences in the amount of histidine-containing substances in the diet of the different groups. When the subjects were infused for 2 h with histamine, resulting in an increase in plasma histamine levels from 0.28 ± 0.04 to 0.71 ± 0.15 ng/ml, the mean hourly excretion of N^{τ}-MH showed no significant increase over the saline control values for the period of the study (Fig. 4). Eight hours following the start of infusion, the cumulative excretion of N^{τ}-MH was 1.44 ± 0.49 μmol for the saline study and 1.34 ± 0.20 μmol for the histamine study. This absence of a significant change in the urinary excretion of N^{τ}-MH is in contrast to the excretion of the further metabolite N^{τ}-MIAA which we have previously shown to increase by approximately 14% during histamine infusion [8].

Since histamine has been implicated as an important mediator in the acute response to inhaled allergen in susceptible subjects, we applied our assay to the measurement of urinary N^{τ}-MH following allergen challenge. In the eight atopic subjects challenged with aerosolised allergen (resulting in a >20% fall in FEV_1), urinary N^{τ}-MH excretion was higher for five of the volunteers but unchanged for the other three (Fig. 5). The values of 254 ± 43 μmol/mol of creatinine (mean ± S.E.M.) on the challenge day and 186 ± 32 μmol/mol of creatinine on the control

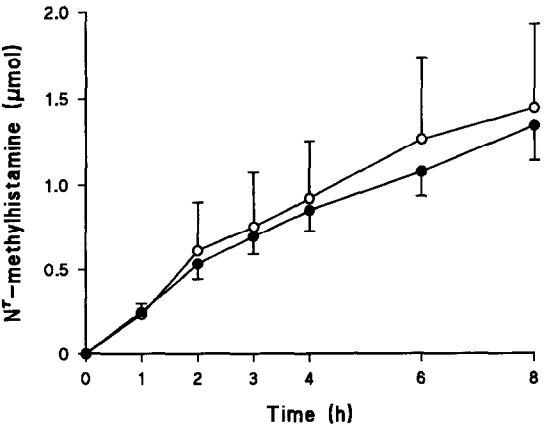

Fig. 4. Cumulative excretion of N^{τ}-MH in urine during and after a 2-h infusion of (●) histamine in saline or (○) saline alone.

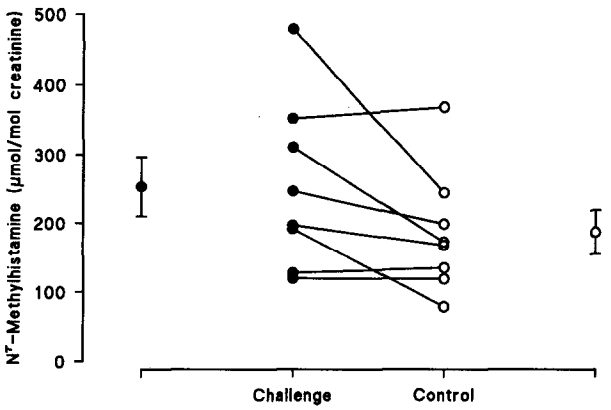

Fig. 5. Excretion of N^{τ}-MH in urine (●) in the 4-h period following challenge with inhaled allergen and (○) under control conditions. The mean values (±S.E.M.) for the two data groups are also shown.

day were not significantly different, although the possibility of at type 2 error cannot be discounted due to the relatively large normal variation in urinary N^{τ}-MH excretion.

In conclusion, these studies suggest that this metabolite is unlikely to be a useful marker of endogenous histamine production in man. It is possible, however, that in disease states such as systemic mastocytosis, which are characterised by abnormal mast cell proliferation, measurement of urinary N^{τ}-MH may be of some use in monitoring mast cell degranulation *in vivo*.

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REFERENCES

1 P. J. Barnes, K. F. Chung and C. P. Page, *Pharmacol. Rev.*, 40 (1988) 49.
2 S. T. Holgate and J. P. Finnerty, *Q. J. Med.*, 66 (1988) 5.
3 P. W. Ind, P. J. Barnes, M. J. Brown, R. Causon and C. T. Dollery, *Clin. Allergy*, 13 (1983) 61.
4 P. W. Ind, M. J. Brown, F. J. M. Lhoste. I. Macquin and C. T. Dollery, *Agents Actions*, 12 (1982) 12.
5 J. J. Keyzer, J. G. R. De Monchy, J. J. van Doormaal and P. C. van Voorst Vader, *N. Engl. J. Med.*, 309 (1983) 1603.
6 J. J. Keyzer, H. Breukelman, B. G. Wolthers, M. van den Heuvel, N. Kromme and W. Chr. Berg, *Agents Actions*, 15 (1984) 189.
7 R. W. Schayer, *Physiol. Rev.*, 39 (1959) 116.
8 I. K. Taylor, G. O'Malley, S. Murray, N. Turner, G. W. Taylor, R. W. Fuller, N. Pride and C. T. Dollery, *J. Appl. Physiol.*, 69 (1990) 591.
9 E. Oosting, J. J. Keyzer and B. G. Wolthers, *Agents Actions*, 27 (1989) 205.
10 Y. Tsuruta, K. Kohashi and Y. Ohkura, *J. Chromatogr.*, 224 (1981) 105.

11 G. Granerus and U. Wass, *Agents Actions,* 14 (1984) 341.
12 A. A. Houdi, P. A. Crooks, G. R. van Loon and C. A. W. Schubert, *J. Pharm. Sci.,* 76 (1987) 398.
13 P. S. Doshi and D. J. Edwards, *J. Chromatogr.,* 176 (1979) 359.
14 J. J. Keyzer, B. G. Wolthers, H. Breukelman, W. van der Slik and K. de Vries, *J. Chromatogr.,* 275 (1983) 261.
15 A. Wollin and H. Navert, *Anal. Biochem.,* 145 (1985) 73.
16 H. Mita, H. Yasueda and T. Shida, *J. Chromatogr.,* 221 (1980) 1.
17 J. J. Keyzer, B. G. Wolthers, F. A. J. Muskiet, H. F. Kauffman and A. Groen, *Clin. Chim. Acta,* 113 (1981) 165.
18 J. K. Khandelwal, L. B. Hough, A. M. Morrishow and J. P. Green, *Agents Actions,* 12 (1982) 583.
19 J. J. Keyzer, H. Breukelman, B. G. Wolthers, F. J. Richardson and J. G. R. de Monchy, *Agents Actions,* 16 (1985) 76.
20 E. Oosting, J. J. Keyzer, B. G. Wolthers and R. J. H. Scholtis, *Agents Actions,* 23 (1988) 307.
21 S. Murray and D. S. Davies, *Biomed. Mass Spectrom.,* 11 (1984) 435.
22 S. Murray and A. R. Boobis, *Biomed. Environ. Mass Spectrom.,* 13 (1986) 91.
23 S. Muray and D. Watson, *J. Steroid Biochem.,* 25 (1986) 255.
24 S. Murray, G. O'Malley, I. K. Taylor, A. I. Mallet and G. W. Taylor, *J. Chromatogr.,* 491 (1989) 15.
25 H. Ehrsson and B. Mellström, *Acta Pharm. Suec.,* 9 (1972) 107.
26 H. Trachtman, A. Tejani, J. J. Keyzer, B. G. Wolthers and E. Oosting, *Nephron,* 47 (1987) 12.