Journal of Chromatography, 307 (1984) 1—9
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2023

REVISED METHOD FOR THE QUANTITATIVE DETERMINATION OF 5-HYDROXYTRYPTAMINE IN HUMAN PLASMA BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY—SELECTED ION MONITORING

SHIGEO BABA*, MASAHIRO UTOH and MASANOBU HORIE

Tokyo College of Pharmacy, 1432-1, Hachioji, Tokyo 192-03 (Japan)

and

YUKIO MORI

Gifu College of Pharmacy, 5-6-1, Mitahora-higashi, Gifu 502 (Japan)

(First received September 20th, 1983; revised manuscript received December 5th, 1983)

SUMMARY

To estimate the 5-hydroxytryptamine level in human plasma by gas chromatography—mass spectrometry—selected ion monitoring (GC—MS—SIM), a recovery method from plasma and derivatization conditions with pentafluoropropionic anhydride were investigated. By heating 5-hydroxytryptamine at 140°C for 4 h in the presence of pentafluoropropionic anhydride the peak intensity of derivatized 5-hydroxytryptamine increased more than three times in comparison with the reported method. There was a marked difference in the plasma levels of 5-hydroxytryptamine obtained using the GC—MS—SIM method compared to those obtained using fluorometric assay.

INTRODUCTION

5-Hydroxytryptamine (5-HT) is generally considered as a neurotransmitter in the brain and may play an important role in sleeping or learning mechanisms [1, 2]. To elucidate the relationship between the plasma level of 5-HT and mental retardation or hereditary disease, numerous studies on the determination of plasma 5-HT level in human have been performed [3—7]. There are, however, large variations in the measured values for plasma 5-HT among these

0378-4347/84/$03.00

reports. A fluorometric assay has been mainly used for the determination of 5-HT in biological samples. In this technique, however, it is difficult to correct for the recovery of 5-HT from plasma and to remove the interference of other compounds having the same fluorescence character as 5-HT. On the other hand, gas chromatography—mass spectrometry—selected ion monitoring (GC—MS—SIM) using deuterated 5-HT as an internal standard has also been applied to the quantitative determination of 5-HT [8, 9] because the instrumentation provides good sensitivity and specificity. However, in these reports, the analyzed samples were limited to urine and brain.

This present paper describes improved analytical methodology for the determination of 5-HT in human plasma by GC—MS—SIM. The method involves derivative formation for GC—MS—SIM analysis using deuterated 5-HT as an internal standard.

EXPERIMENTAL

Chemicals

5-HT[$\alpha,\alpha,\beta,\beta,$-$d_4$] creatinine sulphate (5-[^{2}H]HT) (98.0 atom %) and 5-HT[sidechain-^{14}C] creatinine sulphate (5-[^{14}C]HT) (58.0 mCi/mmol) were purchased from Merck Sharp & Dohm Canada and New England Nuclear, respectively.

Instrumentation

A Shimadzu GC-6 AM gas chromatograph was equipped with a synchronized accumulating radioisotope detector having five counting cells (cell volume = 30 ml) [10]. A Shimadzu LC-2 high-performance liquid chromatograph was also equipped with a synchronized accumulating radioisotope detector having five counting cells (cell volume = 1.1 ml) [11]. Gas chromatography (GC) was carried out under the following conditions: column, 1.5% OV-1 (2 m × 3 mm I.D.); injection port temperature, 220°C; column oven temperature, 200°C; carrier gas flow-rate, 50 ml/min (N_2). High-performance liquid chromatography (HPLC) operated under the following conditions; column, LiChrosorb C_{18} (4.6 mm × 15 cm, 5 μm); elution solvent, 0.01 *M* perchloric acid—methanol (9:1, v/v); flow-rate, 0.6 ml/min. SIM profiles were obtained with a Shimadzu LKB 9000B GC—MS instrument equipped with a multiple ion detector. Fluorometric assay was performed on a Hitachi MPF-2A fluorometer. Excitation and emission wavelengths were set to 380 nm and 500 nm, respectively.

Recovery of 5-HT from human plasma

To 200 μl of human plasma were added 100 nCi of 5-[^{14}C]HT and 5-HT was recovered by the following methods.

Method I. To the plasma sample were added 100 μl of 3% ascorbic acid, 2.5 ml of 2 *M* phosphate buffer (pH 10.5) and 2 ml of *n*-butanol. After shaking the sample for 20 min, the *n*-butanol layer was separated and washed with 0.5 ml of 0.05 *M* ammonium hydroxide. After addition of 2.5 ml of cyclohexane and shaking for 3 min, the upper layer was separated. To the obtained upper layer were added 200 μl of 0.05 *M* formic acid and the mixture was shaken for

5 min. After centrifugation, the aqueous layer was separated. This extraction was carried out once more with 200 μl of 0.05 *M* formic acid.

Method II. To the plasma sample were added 200 μl of 0.1 *M* zinc sulphate and 200 μl of 0.1 *M* barium hydroxide. After centrifugation, the supernatant was separated. To the residue were added 200 μl of 0.1 *M* zinc sulphate and 200 μl of 0.1 *M* barium hydroxide, and the supernatant was obtained after centrifugation.

Method III. To the supernatant described in Method II were added 200 μl of 0.5 *M* borate buffer (pH 10.0) and 5 ml of a mixed solution of *n*-butanol–diethyl ether (1:4, v/v). After shaking for 5 min, the organic layer was separated. This extraction was repeated twice.

Stability of 5-HT during recovery and derivatization processes

A portion of the recovered solutions (corresponding to about 1 nCi) obtained by Method I, II or III was injected into the radio-HPLC system. Each remaining solution was evaporated to dryness. After adding 100 μl of pentafluoropropionic anhydride (PFPA) to the residue, the mixture was heated at 60°C for 3 h. After evaporating the excess PFPA under a nitrogen stream, the residue was dissolved in 20 μl of ethyl acetate. A sample solution containing about 10 nCi of radioactivity was injected into the radio-GC system.

Derivatization conditions of 5-HT with PFPA

After adding 100 μl of PFPA to 200 nCi of 5-[^{14}C]HT, each solution was stood at room temperature, 60°C, 80°C, 100°C, 120°C, 140°C or 160°C. From each reaction mixture, 50 μl of solution were taken at 1, 2, 4 and 6 h after starting the reaction. Each solution was evaporated under a nitrogen stream. To the residue were added 20 μl of ethyl acetate and a portion of this solution (about 9 nCi) was injected into the radio-GC system.

Adsorption of 5-HT derivative on the surface of the GC connecting tube

The injection port was directly connected to a combustion tube by a brass tube (15 cm × 0.1 mm I.D.) to diminish adsorption of derivatized 5-HT on the column packing material. To 640 nCi of 5-[^{14}C]HT were added 100 μl of PFPA and the mixture was heated at 140°C for 2 h. After evaporating the excess PFPA, the residue was dissolved in 100 μl of ethyl acetate and a portion of this solution containing about 25 nCi was injected. $^{14}CO_2$ which was exhausted from the combustion tube was introduced into 6 ml of the mixed solution of methanol–ethanolamine (1:1, v/v). The radioactivity recovered in the ethanolamine was measured with a liquid scintillation counter.

Calibration curve for the GC–MS–SIM method

Appropriate amounts of 5-HT were added to 100 ng of 5-[^{2}H]HT in ratios from 0.1 to 2.0, and 100 μl of PFPA were added to the prepared mixture. After heating at 140°C for 2 h, the excess PFPA was evaporated under a nitrogen stream and the residue was dissolved in 20 μl of ethyl acetate. The multiple ion detector was focused on the ions of *m/z* 451 and 454, and 1 μl of each ethyl acetate solution was injected into the GC–MS system. The peak height ratio was calculated manually from the SIM profiles obtained.

Detection limit

To 5-HT in amounts of 0.1—100 ng were added 100 μl of PFPA and the mixture was heated at 140°C for 2 h. After evaporating the excess PFPA, the residue was dissolved in 200 μl of ethyl acetate and 1—5 μl of these solutions were injected into the GC—MS systems.

Determination of 5-HT in human plasma by GC—MS—SIM

After adding 100 ng of 5-[^{2}H] HT as an internal standard to 200 μl of human plasma, 5-HT was recovered from plasma by Method III. 5-HT was derivatized by adding 100 μl of PFPA and heating at 140°C for 2 h. The excess PFPA was evaporated under a nitrogen stream and the residue was dissolved in 20 μl of ethyl acetate; 2 μl of this solution were injected into the GC—MS system.

Determination of 5-HT in human plasma by fluorometric assay

To 1 ml of human plasma, which was the same sample used for the GC—MS—SIM assay, were added 0.5 ml of 5% disodium EDTA, 5 ml of water, 1 ml of 10% zinc sulphate and 0.5 ml of 1 *M* sodium hydroxide, then the mixture was shaken for 10 min. After centrifugation, 5 ml of the supernatant were added to a mixture of 1.5 ml of 0.1 *M* borate buffer (pH 12.0), 2.5 g of sodium chloride and 7 ml of *n*-butanol. After shaking for 10 min, the *n*-butanol layer was separated and washed with 5 ml of 0.1 *M* borate buffer. To 5 ml of the *n*-butanol layer obtained were added 7.0 ml of *n*-heptane and 1.5 ml of 0.05 *M* phosphate buffer (pH 7.0). After shaking for 5 min, the aqueous layer was separated. To 1 ml of the obtained aqueous solution were added 0.1 ml of 0.1 *M* ninhydrin and 0.1 ml of 0.15% ascorbic acid. The mixed solution was heated at 75°C for 30 min. After cooling to room temperature, fluorescence intensity was measured. The calibration curve was prepared by using a known amount of 5-HT instead of human plasma.

RESULTS AND DISCUSSION

To determine trace amounts of 5-HT in plasma with good accuracy using GC—MS—SIM, it is necessary to increase the recovery of 5-HT from plasma and the derivatization yield. Recovery methods for 5-HT from biological samples for GC analysis that have been used include adsorption with XAD-2 resin [12], deproteinization with zinc sulphate—barium hydroxide [13], or extraction with a polar organic solvent such as *n*-butanol [14]. In these reports, however, no detailed study on percentage recovery or derivatization yield was performed. In this present paper, fundamental investigations on recovery method and derivatization of 5-HT were carried out by use of a radioisotope tracer technique.

The recovery of 5-HT from plasma obtained by Method I, II or III was 63.9, 83.3 or 64.9%, respectively (Table I). To confirm the stability of 5-HT during the recovery procedure, a portion of the solution obtained from plasma by Method I, II or III was injected into the radio-HPLC system. It was apparent that decomposition of 5-HT did not occur during the recovery procedure, because a single peak corresponding to 5-HT appeared in each case. Radio gas chromatograms of the PFPA derivative of 5-[^{14}C] HT recovered from plasma by

TABLE I

RECOVERY OF 5-[^{14}C]HT AFTER PROCESSING FROM PLASMA

Method	Recovery (%)
I: Extraction using *n*-butanol following 0.5 *M* formic acid	63.9 ± 2.7*
II: Deproteinization using 0.1 *M* zinc sulphate and 0.1 *M* barium hydroxide	83.3 ± 1.7
III: Extraction using *n*-butanol—diethyl ether (1:4) after deproteinization	64.9 ± 2.5

*Mean ± S.D. (n = 3).

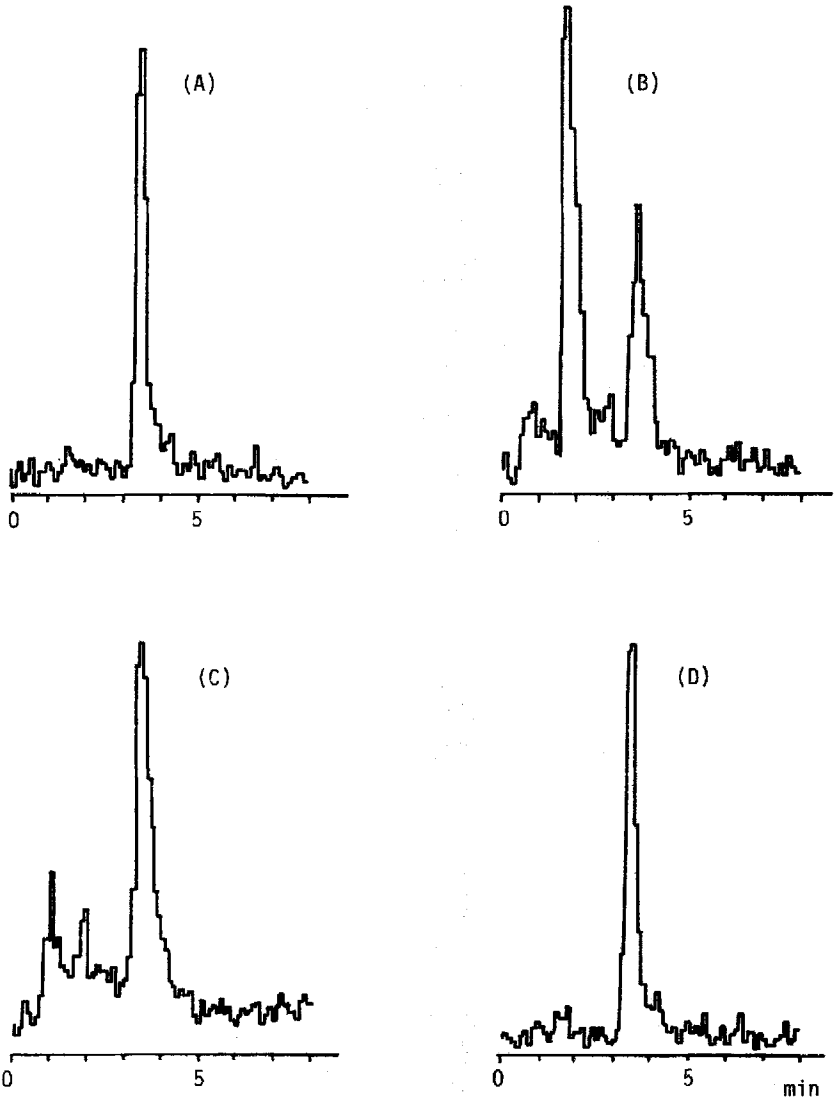

Fig. 1. Radio gas chromatograms of 5-[^{14}C]HT-tri-PFP: authentic (A), and recovered from plasma using Method I (B), Method II (C) and Method III (D).

Method I, II or III following reaction with PFPA according to the method of Beck et al. [9] (60°C, 3 h) are shown in Fig. 1. A single peak corresponding to 5-HT-tri-PFP appeared when Method III was used. On the other hand, peaks other than 5-HT-tri-PFP appeared when Methods I (about 65%) and II (about 40%) were used. When authentic 5-[^{14}C]HT was derivatized with PFPA in the presence of formic acid or zinc sulphate—barium hydroxide, similar peaks originating from the decomposed products appeared. It was apparent that the reagents used in the recovery procedure caused the decomposition of 5-HT in the derivatization reaction.

Equipping the radio-GC system with a synchronized accumulating radioisotope detector makes it possible to increase the detection efficiency without sacrificing resolution power and to obtain the radioactive intensity on the chromatogram in digital form. Thus the ratio of radioactive intensity on the chromatogram to injected radioactivity can be accurately estimated. We named this ratio "GC peak yield". The relationship between GC peak yield and derivatization conditions was examined, and the results obtained are shown in Fig. 2. The GC peak yield of 5-HT-tri-PFP reached a maximum at 140°C and then decreased at 160°C. At lower temperatures, the GC peak yield also increased when a longer reaction time was used. At higher temperatures, however, maximum GC peak yield was attained at 4 h. With the derivatization conditions reported by Beck et al. [9] (60°C, 3 h), the GC peak yield of 5-HT-tri-PFP was only 18%, while raising the reaction temperature to 140°C led to an increase of the GC peak yield to about 51% (reaction time 4 h). These derivatization conditions increased the GC peak yield more than three times in comparison with Beck's conditions. From these results it can be seen that the radio-GC system equipped with synchronized accumulating radioisotope detector is useful for estimating the GC peak yield and finding the best derivatization conditions.

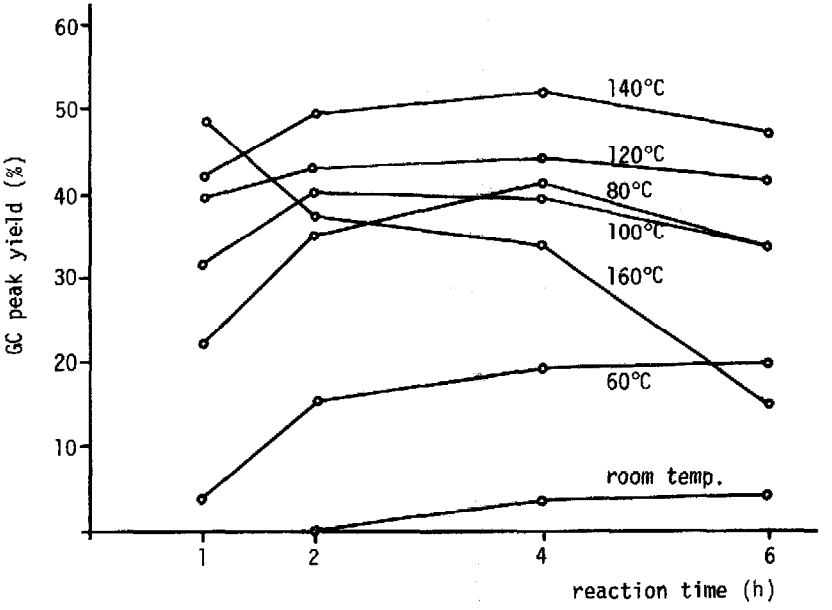

Fig. 2. Relationship between GC peak yield of 5-HT-tri-PFP and derivatization conditions.

To clarify whether the GC peak yield of 5-HT-tri-PFP was identical with the derivatization yield of 5-HT-tri-PFP, the following experiment was performed. An injection port was directly connected to a combustion tube by means of a brass tube, and the $^{14}CO_2$ that was exhausted from the combustion tube was absorbed into ethanolamine [15]. When the column oven temperature was set to 300°C, the recovery of the radioactivity was about 87%. Lowering the oven temperature to 200°C which was used for the assay of 5-HT-tri-PFP led to a decrease of the recovered radioactivity to 53%. It was apparent that the derivatization yield was about 87% or above under the conditions employed (140°C, 4 h), but an appreciable amount of this derivatized compound adsorbed onto the surface of the connecting metal tube. The derivatization of 5-HT for GC—MS—SIM analysis was carried out at 140°C for 2 h, because the GC peak yield reached an approximate equilibrium at 2 h when a temperature of 140°C was used. The calibration curve obtained by the GC—MS—SIM method showed good linearity between the peak height ratio and the molar ratio (r = 0.999). In preparing this calibration curve, the fragment ions m/z 451 and 454 were used as monitoring ions. There was some anxiety that some isotope effects might occur during this fragmentation process. However, the slope of the obtained calibration curve was close to 1. This means that no isotope effect occurred during the fragmentation process. Five picrograms of 5-HT were detected as the tri-PFP derivative with a signal-to-noise ratio of 2.5 (Fig. 3). When 200 μl of human plasma were used for the sample, the amount of derivatized 5-HT injection into the GC—MS system was estimated to be about a few nanograms. It was apparent that this method has an enough sensitivity to measure the 5-HT content in human plasma.

Quantitation of 5-HT in biological samples has mainly been performed by fluorometric assay. However, in this technique, 5-HT is not chromatographically separated and there is the possibility that compounds other than 5-HT affect the fluorescence intensity originating from 5-HT. In order to compare the analytical accuracy of the GC—MS—SIM method with that of the fluorometric assay, 5-HT was measured in a human plasma sample. Several fluorometric assays for 5-HT have been reported [16—18]. Of these, the

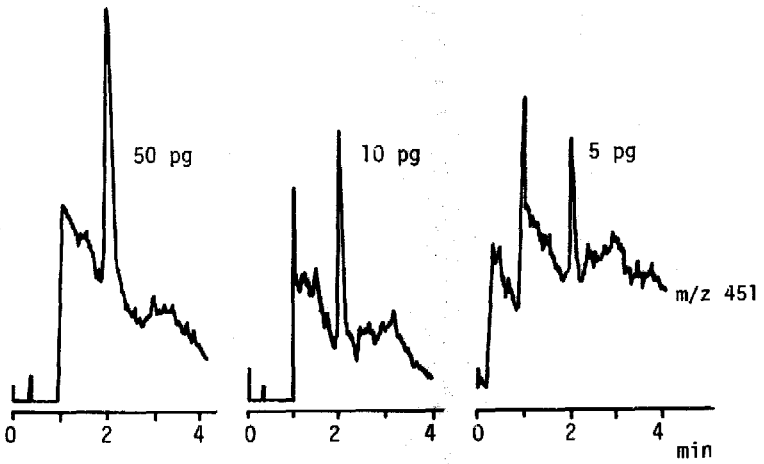

Fig. 3. Detection limit of 5-HT-tri-PFP by selected ion monitoring.

TABLE II

MEASUREMENT OF 5-HT IN HUMAN PLASMA BY GC—MS—SIM AND FLUOROMETRIC ASSAY

	GC—MS—SIM (ng/ml plasma)*	Fluorometric assay (ng/ml plasma)
1	307.8 ± 10.2	221.7
2	294.7 ± 6.7	191.2
3	281.2 ± 6.9	210.0
4	289.9 ± 4.7	169.1
5	301.6 ± 6.9	213.8
Mean ± S.D.	295.0 ± 9.2	201.2 ± 18.9
C.V. (%)	3.1	9.4

*Each value represents the mean ± S.D. of triplicate measurements.

ninhydrin method might be the most sensitive and has been used for clinical diagnosis. In this paper, the ninhydrin method was used.

The 5-HT level in human plasma was found to be 295.0 ± 9.2 ng/ml by the GC—MS—SIM method and 201.2 ± 18.9 ng/ml by the fluorometric assay (Table II), the former value being about 1.5 times higher than the latter. One of the reasons why the fluorometric assay showed a lower value might be due to the different extraction ratio of 5-HT from plasma and from an authentic sample solution which was used for the preparation of the calibration curve. A fixed amount of 5-[^{14}C]HT was added to the plasma sample or aqueous solution and 5-HT was extracted by the method used for the fluorometric assay. The recovery of radioactivity was 15.6 ± 0.1% for the aqueous solution and 13.3 ± 0.2% for the plasma sample. The addition of plasma lowered the recovery of radioactivity by about 15%. There is thus a possibility that lower values might be obtained when the calibration curve made 5-HT aqueous solution is used for measuring 5-HT in plasma. This phenomenon also suggests that a low extraction ratio is responsible for the low sensitivity in the fluorometric assay, because only 10% of radioactivity was recovered in the aqueous layer. The other reason why the fluorometric assay showed a lower assay value might be due to a quenching phenomenon which originates from biological compounds other than 5-HT. However, there is no evidence for this quenching phenomenon. A GC—MS—SIM method using 5-HT labelled with a stable isotope can automatically correct for the losses of 5-HT during the recovery and derivatization processes. The coefficients of variation were 3.1% for the GC—MS—SIM method and 9.4% for the fluorometric assay. This means that the GC—MS—SIM method has a better accuracy.

ACKNOWLEDGEMENTS

We are greatly indebted to Miss M. Akahane and Miss M. Shimokawa for skilled technical assistance.

REFERENCES

1 J. Matsumoto and M. Jouvet, C.R. Soc. Biol., 158 (1964) 2137.
2 M. Jouvet, Science, 163 (1969) 32.
3 R.M. Hardisty and R.S. Stacey, J. Physiol. (London), 130 (1955) 711.
4 J. Stratigos, A. Katsambas and P. Glanopoulou, Brit. J. Dermatol., 90 (1974) 451.
5 C.M.B. Pare, M. Sandler and R.C. Stacey, J. Neurol. Neurosurg. Psychiat., 23 (1960) 341.
6 F. Rosner, B.H. Ong, R.S. Paine and D. Mahanand, Lancet, i (1965) 1191.
7 J. Berman, M. Hulten and J. Lindsten, Lancet, i (1967) 730.
8 H.-Ch. Curtius, H. Farner and F. Rey, J. Chromatogr., 199 (1980) 171.
9 O. Beck, F.-A. Wiesel and G. Sedvall, J. Chromatogr., 134 (1977) 407.
10 S. Baba and Y. Kasuya, J. Chromatogr., 196 (1980) 144.
11 S. Baba, M. Horie and K. Watanabe, J. Chromatogr., 244 (1982) 57.
12 J. Segra, F. Artigas, E. Martinez and E. Gelpi, Biomed. Mass Spectrom., 3 (1976) 91.
13 F. Cattabeni, S.H. Koslow and E. Costa, Science, 178 (1972) 166.
14 I.L. Martin and G.B. Ansell, Biochem. Pharmacol., 22 (1973) 521.
15 S. Baba, T. Konishi and H. Ido, Yakugaku Zasshi, 93 (1973) 532.
16 S. Udenfriend, H. Weissbach and B.B. Brodie, Methods Biochem. Anal., 6 (1958) 95.
17 D.F. Bogdanski, A. Pletscher, B.B. Brodie and S. Udenfriend, J. Pharmacol. Exp. Ther., 117 (1956) 82.
18 S.H. Snyder, J. Axelrod and M. Zweig, Biochem. Pharmacol., 14 (1965) 831.