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EVALUATION OF PLASMA 3-METHOXY-4-HYDROXYPHENYLGLYCOL

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SUMMARY

A high-performance liquid chromatographic (HPLC) assay suitable for the evaluation of
3-methoxy-4-hydroxyphenylglycol (MHPG) in 1-ml aliquots of plasma is presented.
Preparatory steps include extraction into ethyl acetate and minicolumn chromatography.
Recoveries are monitored with [^{3}H]MHPG. The HPLC procedure utilizes a C_{18} column,
isocratic elution and amperometric detection. The assay was checked against a gas chromato-
graphic—mass spectrometric procedure; the two procedures correlated well with a correla-
tion coefficient of 0.99. Intra-assay reproducibility was 5.4%, inter-assay reproducibility
10.7%. Immediate changes in the orthostatic position did not affect the plasma MHPG
concentration. Based on 22 normal controls the daytime plasma MHPG level was 2.98 ± 0.66
ng/ml (mean ± S.D.).

INTRODUCTION

The noradrenergic system has been implicated in a variety of psychiatric
disorders. Much of this work has been on depression, but also psychoses
have been studied, and anxiety disorders are a rapidly growing area of

interest. The obvious difficulties of directly assessing noradrenergic function in the intact brain have led to a variety of alternative strategies. Measurement of plasma or urinary catecholamines has been widely used, but plasma half-lives are only a few minutes, and levels are sensitive to posture, diet and emotional arousal [1—4]. The search for a more stable indicator of noradrenergic function led quickly to 3-methoxy-4-hydroxyphenylglycol (MHPG), the major metabolite of norepinephrine in cerebrospinal fluid (CSF) [5]. The demonstration that cerebral venous blood has higher levels of MHPG than arterial blood supports the use of MHPG as an indicator of central noradrenergic activity [6]. Elsworth et al. [7] have shown that plasma MHPG levels in vervet monkeys are highly correlated (r = 0.94–0.99) with brain MHPG directly measured in a variety of areas, thus validating much of the research that has been done on and the conclusions drawn from MHPG measurements.

Recent studies have revealed a complex picture of MHPG transport and metabolism [8]. It is water-soluble, and it should not therefore be surprising that it is transported to the CSF from the periphery as well as vice versa. A two-compartment model has been used to describe the system [9]. Previous estimates of the proportion of plasma MHPG that originates in brain were elevated because it was not recognized that half of plasma MHPG is metabolized to vanilmandelic acid. Kopin et al. [9] suggest that less than 25% of plasma MHPG originates in brain. Despite these reconsiderations regarding the source of plasma MHPG, it remains the single most useful measure of sympathetic activity.

The majority of psychiatric studies use a between-subjects design to identify differences between groups of patients and normals, and the stability of MHPG levels avoids much of the rapid individual variation that characterizes catechol amine measurements. The half-life of MHPG in plasma is approx. 30 min. Standing and mental stress do not influence plasma MHPG levels in periods shorter than 20 min [10]. Acute administration of caffeine does not appear to change plasma MHPG levels [11].

In manic depressive patients, urinary MHPG levels are lower during depression and higher during mania [12]. Urinary MHPG levels are reduced in patients with endogenous depression associated with bipolar disorders as compared to normals and to patients with non-endogenous depressions [13]. Low levels of urinary MHPG may predict response to imipramine treatment [14]. Long-term benzodiazepine treatment is associated with substantial reductions in urinary MHPG, which return toward normal during drug withdrawal [15]. Plasma free MHPG is highly correlated (r = 0.59) with self-ratings of state anxiety in normal subjects [16]. Plasma free MHPG increases in response to administration of an α_2-adrenergic blocker, yohimbine, and these increases are higher in patients with anxiety disorders than in normals [17].

MHPG measurements offer an important tool for psychiatry, and accurate, fast and efficient methods to measure it are of obvious importance. However, simple reliable plasma or serum assays for MHPG are scarce. The method of choice for plasma MHPG has been a combination of gas chromatography- mass spectrometry (GC—MS) [18, 19]. A few high-performance liquid chromatography (HPLC) assays for MHPG have been described for urine [20, 21] and

CSF [22, 23]. A few HPLC assays for plasma MHPG concentrations have been published [24—28]. We are describing an alternative, easily performed and reproducible HPLC procedure for plasma MHPG which boasts simple preparatory steps allowing pre-processing of twelve samples within less than 2 h.

EXPERIMENTAL

Materials

Tritiated MHPG was prepared in-house as described below. From Sigma (St. Louis, MO, U.S.A.) we obtained 3,4-dihydroxyphenylglycol, MHPG and Na_2EDTA. A sample of 3-hydroxy-4-methoxyphenylglycol (iso-MHPG) was a generous gift from Dr. H.J. Gärtner (Eberhard-Karls-Universität, Tübingen, F.R.G.).

Catechol-O-methyl transferase (COMT) was prepared in-house from rat liver [29]. It has become available commercially as well (Upjohn, Kalamazoo, MI, U.S.A.).

Tritiated S-adenosyl-L-methionine (9—15 Ci/mol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Tris buffer (500 mM) was made up to contain 150 mM magnesium chloride, 15 mM ethyleneglycoltetraacetic acid (EGTA) and 4.9 mM glutathione (all from Sigma) and 0.4 mM benzyloxyamine (Aldrich, Milwaukee, WI, U.S.A.). HPLC-grade ethyl acetate was used for various extractions. For preliminary separation of [^{3}H]MHPG, Merck silica thin-layer chromatography (TLC) plates, 0.25 mm thickness, 20 × 20 cm (American Scientific Products, Romulus, MI, U.S.A.) were used. The TLC developing solution was propanol-2— ammonium hydroxide— water (16:1:3).

Heparinized vacutainers or catecholamine collection tubes containing 6 mg glutathione and 9 mg EGTA per 5 ml blood [30] were used.

Reagents for the extraction of MHPG from plasma included 0.9% sodium chloride solution, 0.1 M acetic acid and water-saturated ethyl acetate.

A DuPont Type OD cartridge (DuPont, Wilmington, DE, U.S.A.) was used as a minicolumn in the preparatory steps. The pH 4.15 elution buffer for this step consisted of 89 mM sodium acetate containing 13.6% methanol. The HPLC step utilized a 30 cm × 3.9 mm μBondapak C_{18} column, 10 μm particle size (Waters Assoc., Milford, MA, U.S.A.), an LC-4A amperometric detector (BioAnalytical Systems, West Lafayette, IN, U.S.A.) and a pH 4.15 mobile phase consisting of 89 mM sodium acetate containing 4% methanol and 0.1 mM EDTA.

Methods

Synthesis of radioactive MHPG to monitor recoveries. The synthesis of radioactive MHPG was based on, and analogous to, the 3-O-methylation step of the single isotope derivative assay for catecholamines [31]. 3,4-Dihydroxyphenylglycol (1 μg), 0.4 ml Tris buffer, 0.1 ml COMT and 0.3 ml tritiated S-adenosyl-L-methionine were incubated for 2.5 h at 37°C. The reaction was stopped by addition of 0.1 ml of 0.1 M EDTA. MHPG, tritium-labeled in the 3-O-methyl position, as well as iso-MHPG, labeled in the 4-O-methyl position, were extracted with 3 × 4 ml water-saturated ethyl acetate.

The fractions were pooled and evaporated under nitrogen. The residue was reconstituted in 200 μl methanol, spotted on a silica TLC plate and developed in the TLC developing solution. The area where an authentic MHPG standard migrated was eluted with 2 × 3 ml methanol.

The eluates were combined, evaporated and reconstituted in 200 μl mobile phase; 100 μl were injected onto the HPLC column under the assay conditions elaborated below. Fractions of 0.5 ml were collected and those eluting with authentic MHPG were pooled. A small aliquot of the pooled [^{3}H]MHPG was reinjected onto the HPLC column and specific activity calculated from peak height and radioactivity. Iso-MHPG, which traveled with MHPG on the TLC system described here, was separated by the HPLC step. Labeled iso-MHPG could, therefore, be obtained from the same procedure by collecting the appropriate fraction(s) from the preparatory HPLC step. The labeled MHPG was stable for at least a year at −20°C. It could be repurified readily on an HPLC system, should the need arise.

Extraction of plasma MHPG. To 1 ml plasma, 5000 cpm [^{3}H]MHPG and 0.5 ml of sodium chloride solution were added. The pH was adjusted to 5.0 with 0.1 M acetic acid. The mixture was extracted on a multi-sample vortex (SMI, Emeryville, CA, U.S.A.) for 2 × 10 min, each time with 5 ml of water-saturated ethyl acetate. The two supernatants were pooled in a clean glass tube and evaporated under reduced pressure. The residue was reconstituted in 200 μl of water and applied to a preparatory minicolumn which had been activated with 2 ml methanol followed by 2 ml mobile phase. The minicolumn was placed in the Prep I (DuPont De Nemours, Wilmington, DE, U.S.A.) which was used to wash with 0.25 ml mobile phase, then to elute the MHPG with 0.6 ml of elution buffer and finally to dry this eluate. The residue obtained from the Prep I was reconstituted in 200 μl mobile phase, 25 μl of which were counted in Bray's solution to determine extraction recoveries and 100 μl of which were injected onto the HPLC column.

HPLC conditions. Separation and quantitation of MHPG was achieved isocratically on a μBondapak C$_{18}$ column with the mobile phase. A Model 110A Altex pump (Anspec, Ann Arbor, MI, U.S.A.) was used to adjust the flow-rate to 1 ml/min, and the column effluent was monitored with an amperometric detector equipped with a glassy carbon electrode. The electrochemical detector was set at +0.73 V applied potential and 1 nA range. While we used room temperature to run MHPG on the HPLC system, that room temperature should be controlled to range between 18 and 24°C. A five-point standard curve of MHPG in mobile phase was prepared daily by adding 2.5- 20 ng MHPG to 1-ml aliquots of mobile phase. Aliquots of 100 μl of these solutions were injected directly onto the HPLC column.

Collection of normal controls. Ten healthy volunteers (five men and five women), who had given written consent, were asked to lie supine. A butterfly needle fitted with a heparin lock was inserted immediately into the anti-cubital vein and six blood samples of 7 ml each were drawn at 10-min intervals for 50 min. At that time the subjects were asked to stand for 15 min and a final blood specimen was drawn. From an additional twelve individuals (five men and seven women) blood samples were drawn seated using simple venipuncture. All samples were processed immediately and the plasma was frozen at - 20°C until analysis.

To check the accuracy of our assay eight plasma samples ranging from 2 to 15 ng/ml were also analyzed by GC—MS, courtesy of Dr. David C. Jimerson (National Institute of Health, Mental Health Division, Bethesda, MD, U.S.A.). The samples were selected and MHPG was added to the last two to give a broad range of values for the comparison study.

Inter-assay reproducibility was evaluated by inclusion of an individually frozen aliquot obtained from one of four plasma pools in each separate assay and of thirteen samples, each run in more than one assay. Each plasma pool was assayed in eight to nineteen separate assays. Intra-assay reproducibility was evaluated from the variation encountered between 22 duplicate samples assayed within specific runs, frequently at the beginning and end of a given run. Recovery of added MHPG was based on the standard curves generated from plasma to which known quantities of authentic MHPG ranging from 0.5 to 20 ng/ml were added.

RESULTS

Methylation of 3,4-dihydroxyphenylglycol by [³H] S-adenosyl-L-methionine and COMT resulted in a mixture of compounds from which MHPG and iso-MHPG were resolved by extraction followed by TLC and then HPLC. Under
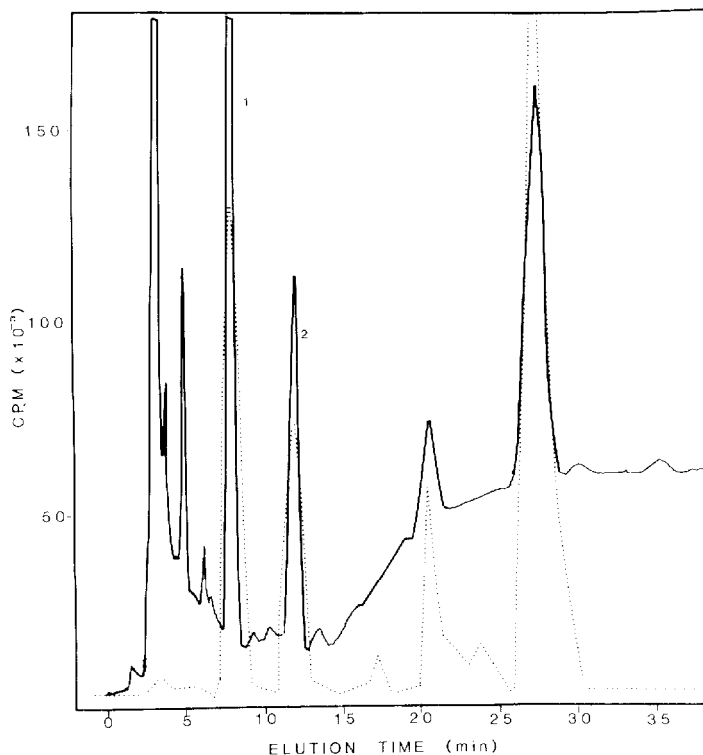

Fig. 1. HPLC profile of the partially purified products derived from the COMT methylation of 3,4-dihydroxyphenylglycol with tritiated S-adenosyl-L-methionine. The solid line is the elution pattern as monitored with an amperometric detector and a glassy carbon electrode. The dotted line represents tritium counts obtained for the corresponding effluents.

the conditions used MHPG and iso-MHPG together with their tritiated analogues were isolated from the C_{18} column at 8 and 12 min, respectively (Fig. 1). The specific activity of various [^{3}H] MHPG preparations produced by this method in our laboratory was of the order of 7 μCi/nmol.

Chromatograms obtained from the analytical HPLC assay of standards and plasma MHPG are shown in Fig. 2. The assay is linear for injections ranging from 0.25 to 2 ng per 100 μl or from 2.5 to 20 ng/ml. Recoveries of MHPG through the preparatory steps of the procedure, based on the recovery of radioactive tracer, range from 25 to 35%. The percentage recovery can be increased by increasing the amount of ethyl acetate used for extraction; however, at the time this paper was written we were interested in maintaining the lowest possible volume for the subsequent evaporation step. Using this radioactive tracer to correct for losses the overall assay recovery of MHPG, added in 0.5–10 ng/ml concentrations to plasma, is 100 ± 2.2% (mean ± standard error of the mean, n = 21).
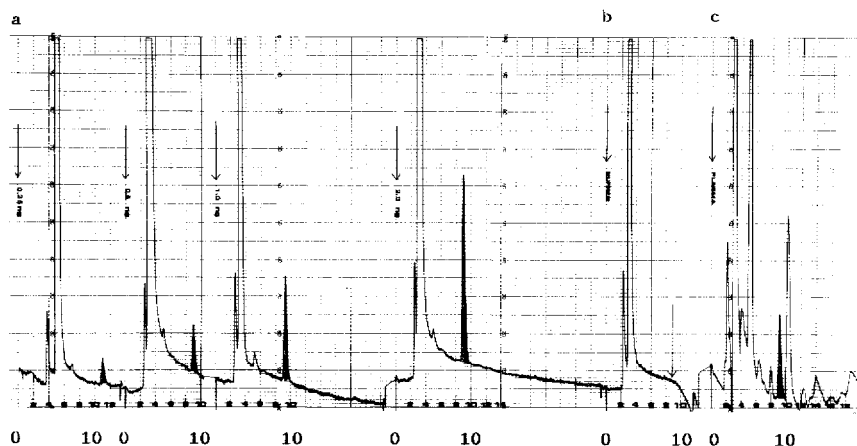

Fig. 2. HPLC profiles of (a) MHPG standards (0.25, 0.5, 1.0 and 2.0 ng), (b) buffer only and (c) plasma.

Eight duplicate plasma samples ranging from 2 to 15 ng/ml assayed by Dr. David C. Jimerson utilizing GC—MS, gave a correlation of 0.99 with the HPLC procedure as shown in Table I. Even with the omission of the 14.7 ng/ml value, a correlation of 0.94 was obtained. Slope of the regression curve for the two methods is 0.90 indicating slightly, albeit not statistically significant, higher values by the HPLC procedure.

The intra-assay coefficient of variation was 5.4% (n = 14), the inter-assay coefficient of variation 10.7% (n = 29). The limit of sensitivity of the assay was < 125 pg per injection or approx. 1.25 ng/ml of plasma.

Unlike plasma norepinephrine, plasma MHPG is relatively independent of the posture which has been assumed for blood collection. Based on an univariate Anova analysis, samples drawn immediately and 10 min after subjects assumed the supine position (samples A and B, Table II) are not significantly different in MHPG concentration than one drawn 50 min later (sample F, Table II). Plasma concentrations obtained after 15 min of standing (sample

TABLE I

COMPARISON OF PLASMA MHPG LEVELS DETERMINED BY GC—MS AND HPLC

Values are expressed in ng/ml. The correlation coefficient, r, is 0.989 ($n = 8$).

GC—MS	HPLC
4.4	5.1
3.4	3.8
4.1	4.0
4.0	4.8
5.7	5.9
5.6	5.0
7.6	8.5
15.6	14.7

TABLE II

IMMEDIATE EFFECT OF ORTHOSTATIC POSITION ON PLASMA MHPG CONCENTRATIONS

Based on a univariate Anova analysis (MIDAS, University of Michigan MTS system) there is no significant difference between these plasma MHPG values.

Sample		Plasma MHPG concentration (mean ± S.D., $n = 10$) (ng/ml)
A.	Immediately after lying down	3.02 ± 0.70
B.	10 min supine	2.71 ± 0.67
C.	20 min supine	2.48 ± 0.51
D.	30 min supine	2.67 ± 0.48
E.	40 min supine	2.80 ± 0.34
F.	50 min supine	2.60 ± 0.67
G.	15 min after standing up	2.85 ± 1.16

G, Table II) are no different than the final supine ones (sample F, Table II).

The mean normal value for daytime plasma MHPG based on 22 healthy adults was 2.98 ± 0.66 ng/ml (mean ± S.D.). No significant difference was evident between males and females in this limited population, nor was there an age-related difference discernable in this small group.

DISCUSSION

The assay for MHPG described here is based on a relatively simple procedure. The use of a radioactive internal standard for the evaluation of MHPG recovery through the preparatory procedure was more satisfactory than the use of the traditional HPLC internal standard. Alternative internal standards which were considered included 3-hydroxybenzyl alcohol and iso-MHPG. Of the compounds tested, iso-MHPG appeared to be the most suitable; however, in a few plasma samples an interfering peak was noted on the HPLC profile, even with this compound. On the other hand, the use of tritium labeling allows accurate

corrections to be made for each individual sample for the substantial losses incurred in the preparatory steps. Subsequent errors owing to the HPLC procedure were negligible in our hands. The mass of MHPG added with the tritium label must be subtracted from the final answer.

The DuPont Prep 1 with its OD cartridge works well for the clean-up and concentration of the sample prior to HPLC. The use of these reversed-phase minicolumns reduces the load of unwanted chemicals which are placed on the HPLC column substantially. This allows for the 12-min HPLC running time per sample. In laboratories which do not have the appropriate centrifuge available, minicolumns can be eluted rapidly using vacuum pump systems.

The HPLC elution times, as with all other HPLC methods, are a function of the composition of the mobile phase, column packing, the age of the column and the temperature at which the column is eluted. The unidentified peak which normally elutes at 10– 11 min is much more sensitive to changes in temperature than MHPG. If the ambient temperature rises above 24– 25°C, this peak will elute rapidly enough to interfere with the MHPG peak. The C_{18} column used needs regular weekly rinsing with 100% methanol; however, given columns have been good for more than 650 injections in our hands.

A standard curve is run daily since, although the electrochemical detector response to the MHPG is linearly proportional, the recorded peak height is not necessarily in a 1:1 relationship to the concentration of MHPG, i.e. a plot of peak height versus MHPG concentration yields a straight line whose slope is not necessarily 1.

The measure for MHPG is substantially more robust than that for norepinephrine or epinephrine. The physiological levels of MHPG change more slowly than those of their catecholamine precursors. From a purely chemical point of view MHPG is more stable than norepinephrine or epinephrine because the ring catechol groups have been stabilized by methylation of the 3-OH position and the glycol side-chain is less reactive than the amine one. In addition, the plasma MHPG concentrations are an order of magnitude higher than those of plasma norepinephrine and two orders of magnitude higher than those of plasma epinephrine.

The evidence today suggests that there is an interaction or some sort of linkage between the brain adrenergic systems and the functioning of peripheral catecholamine neurons, particularly those of the sympathetic nervous system [32]. The assay of plasma MHPG may therefore be of considerable utility when there is a need to evaluate catecholamine status.

REFERENCES

1 M. Esler, G. Jackman, A. Bobit and J. Jennings, Life Sci., 25 (1970) 1461.
2 N.J. Christensen and O. Brandsborg, Eur. J. Clin. Invest., 3 (1973) 299.
3 F.C. Luft, L.I. Rankin, D.P. Henry, R. Bloch, C.E. Grim, A.E. Weyman, R.H. Murray and M.H. Weinberger, Hypertension, 1 (1979) 261.
4 R. Nesse, O. Cameron, G. Curtis, D.S. McCann and M.J. Huber-Smith, Arch. Gen. Psychiatry, 41 (1984) 771.
5 J.W. Maas (Editor), MHPG: Basic Mechanisms and Psychopathology, Academic Press, New York, 1983.
6 J.W. Maas, S.E. Hattox, N.M. Greene and D.H. Landis, Science, 205 (1979) 1025.

7 J.D. Elsworth, R.H. Roth and D.E. Redmond, Jr., Brain Res., 235 (1981) 115.
8 I.J. Kopin, D.C. Jimerson, S.P. Markey, M.H. Ebert and R.J. Polinsky, Pharmaco-psychiatry, 17 (1984) 3.
9 I.J. Kopin, E.K. Gordon, D.C. Jimerson and R.J. Polinsky, Science, 219 (1983) 73.
10 P. Hjemjahl, B. Sjoquist, M. Daleskog and K. Eliasson, Acta Physiol. Scand., 115 (1982) 507.
11 D.S. Charney, M.P. Galloway and G.R. Heninger, Life Sci., 35 (1984) 135.
12 K. Greenspan, J.J. Schildkraut, E.K. Hordon, L. Baer, M.S. Aronoff and J. Durell, Psychiatric Res., 7 (1970) 171.
13 J.J. Schildkraut, B.A. Keeler, M. Papousek and E. Hartmann, Science, 181 (1973) 762.
14 D.P. Van Kammen, in J.W. Maas (Editor), MHPG: Basic Mechanisms and Psychophato-logy, Academic Press, New York, 1983, p. 167.
15 H. Petursson, P.A. Bond, B. Smith and M.H. Lader, Biol. Psychiatry, 18 (1983) 207.
16 T.W. Uhde, L.J. Liever, R.M. Post. D.C. Jimerson, J. Boulenger and M.S. Buchsbaum, Psychopharmacol. Bull., 18 (1984) 129.
17 D.S. Charney, G.R. Heninger and A. Brier. Arch. Gen. Psychiatry., 41 (1984) 751.
18 D.C. Jimerson, S.P. Markey, J.A. Oliver and I.J. Kopin, Biomed. Mass Spectroscopy, 8 (1981) 256.
19 S.E. Hattox, in J.W. Maas (Editor), MHPG: Basic Mechanism and Psychopathology, Academic Press, New York, 1983, p. 69.
20 P. Moleman and J.J.M. Borstrok, J. Chromatogr., 227 (1982) 391.
21 M.H. Joseph, B.V. Kadam and D. Risby, J. Chromatogr., 226 (1981) 361.
22 P.J. Langlais, W.J. McEntee and E.D. Bird, Clin. Chem., 26 (1980) 786.
23 G.M. Anderson, J.G. Young, D.J. Cohen, B.A. Shaywitz and D.K. Batter, J. Chromato-gr., 222 (1981) 122.
24 H. Ong, F. Capet-Antonini, N. Yamaguchi and D. Lamontagne, J. Chromatogr., 233 (1982) 97.
25 F. Karege, J. Chromatogr., 311 (1984) 361.
26 A. Minegishi and T. Ishizaki, J. Chromatogr., 311 (1984) 51.
27 P.A. Shea and J.B. Howell, J. Chromatogr., 306 (1984) 358.
28 M. Schsinin, W.H. Chang, D.C. Jimerson and M. Linnoila, Anal. Biochem., 132 (1983) 165.
29 J. Axelrod, Methods Enzymol., 5 (1962) 748.
30 D.S. McCann and M.J. Huber-Smith, J. Clin. Immunoassay, 6 (1983) 308.
31 P.G. Passon and J.D. Peuler, Anal. Biochem., 51 (1973) 618.
32 J.W. Maas and J.F. Leckman, in J.W. Maas (Editor), MHPG: Basic Mechanisms and Psychopathology, Academic Press, New York, 1983, p. 41.