Journal of Chromatography, 427 (1988) 9–17
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4103

TRITIUM ISOTOPE EFFECT IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF DOPAMINE AND DIHYDROXYPHENYLACETIC ACID

JACQUES A. VAN DER KROGT*, CEES F.M. VAN VALKENBURG and RENÉ D.M. BELFROID

Department of Pharmacology, Medical Faculty, University of Leiden, P.O. Box 9503, 2300 RA Leiden (The Netherlands)

(First received November 6th, 1987; revised manuscript received December 17th, 1987)

SUMMARY

A tritium isotope effect has been demonstrated in the high-performance liquid chromatographic analysis of dopamine and its acidic metabolite dihydroxyphenylacetic acid. The chromatographic system consisted of tributyl-*n*-phosphate, bound to a ChromSpher C_8 column, as stationary phase, and a citrate buffer, containing the ion-pairing agent perchlorate, as the mobile phase. For detection we used continuous electrochemical monitoring (for the total amount of solutes) and discontinuous liquid scintillation counting (for radiolabelled molecules) of the column effluent. [^{3}H]Dopamine and [^{3}H]dihydroxyphenylacetic acid were biosynthesized by incubation of homogenates of striatal tissue from rat brains with ^{3}H-labelled L-tyrosine. The tritium-labelled compounds were eluted before the corresponding unlabelled analogues. The capacity factor reduction increased with the number of tritium atoms incorporated in the molecules: for single, double and triple tritium-labelled dopamine the separation factors amounted to 1.015, 1.028 and 1.033, respectively. No isotope separation was observed for 7-^{14}C-labelled dopamine and dihydroxyphenylacetic acid. The isotope effect observed is ascribed to a decrease in lipophilicity following tritium substitution for hydrogen.

INTRODUCTION

In chromatographic analysis of material containing isotopically labelled solutes, one has always to be aware of the possibility of (partial) resolution of the isotopically labelled molecules from the unlabelled mass of the solute. This phenomenon, mostly described as an "isotope effect", has been observed in various analytical systems and for various types of compound (for review see ref. 1).

For our in vivo studies of metabolic dopamine (DA) compartmentation in rat brain, we decided to monitor the specific activities of DA and its metabolites (in particular dihydroxyphenylacetic acid, DOPAC) after a pulse injection of tri-

0378-4347/88/$03.50

tium-labelled L-tyrosine, the precursor of DA [2]. For this purpose we developed a reversed-phase, liquid–liquid partition high-performance liquid chromatographic (HPLC) separation method, combined with electrochemical detection (ED) and liquid scintillation counting (LSC) [3]. During routine application of this procedure evidence was obtained for a substantial isotope fractionation, thus introducing the possibility of erroneous specific activity calculations.

In this paper we report the results of experiments aimed at ascertaining this tritium isotope effect. By examining the effect of the incorporation of more than one tritium atom into DA and DOPAC and of the incorporation of ^{14}C into these molecules, the observed isotope effect was further characterized.

EXPERIMENTAL

Chemicals

The following radioactively labelled compounds were purchased from Amersham (Amersham, U.K.): L-[3,5-^{3}H]tyrosine (specific activity 48 and 46.3 Ci/mmol), L-[2,6-^{3}H]tyrosine (specific activity 41 Ci/mmol), L-[2,3,5,6-^{3}H]tyrosine (specific activity 71 Ci/mmol), [7-^{14}C]dopamine (3,4-dihydroxyphenyl[2-^{14}C]ethylamine, specific activity 50 mCi/mmol).

Reagent-grade chemicals and drugs were from Baker (Deventer, The Netherlands), except for tributyl-*n*-phosphate (Janssen, Beerse, Belgium), and dopamine hydrochloride and dihydroxyphenylacetic acid (Fluka, Buchs, Switzerland).

All solutions were made with glass-distilled deionized water.

HPLC analysis

HPLC analysis was performed as described before [3,4], with some modifications. The liquid chromatograph was a Spectra-Physics SP8770 (Santa Clara, CA, U.S.A.), equipped with a Valco injector (Houston, TX, U.S.A.) with a 500-μl sample loop. The chromatographic separation was brought about in a stainless-steel ChromSpher C_8 column (5-μm particles, 250 mm$\times$4.6 mm I.D.; Chrompack, Middelburg, The Netherlands), which was loaded with the stationary phase tributyl-*n*-phosphate (TBP). The mobile phase consisted of 0.05 *M* sodium citrate, 0.2 *M* sodium perchlorate and 1.5 m*M* NaEDTA, had a final pH of 4.9 and was saturated with TBP. The flow-rate of the mobile phase was 0.4 or 0.5 ml/min, and the temperature 21.2°C.

For analysis, 0.5 ml of the perchlorate preparation described below was injected into the chromatograph. Quantification of total (unlabelled and labelled) DA and DOPAC was performed with a laboratory-made thin-layer electrochemical detector, the potential of which was maintained at 800 mV. For quantification of the radiolabelled compounds by LSC, the DA and DOPAC regions of the HPLC effluent were collected from the detector cell in 0.33- or 0.5-min portions (yielding 0.13–0.25 ml fractions) in counting vials. The moment of vial change was registered on the recording of the ED signal by way of a footswitch signal. After addition of 5 ml of Picofluor 15 (Packard, Groningen, The Netherlands) to each vial, single or dual label counting was performed in a Tri-Carb Model 2409 liquid scintillation counter (Packard, Downers Grove, IL, U.S.A.).

Sample preparation

Striatal tissue of male Wistar rats, weighing 200–250 g, was homogenized in a modified Krebs–Ringer solution, using an ice-cooled PTFE–glass Potter–Elvehjem homogenizer. Portions of the homogenate were incubated in the presence of ^{3}H-labelled L-tyrosine or ^{14}C-labelled DA, the final incubation conditions being 125 m*M* sodium chloride, 5 m*M* potassium chloride, 1 m*M* magnesium chloride, 1 m*M* calcium chloride, 10 m*M* glucose, 1 μ*M* L-tyrosine, 15 m*M* sodium phosphate (pH 6.2), 1–3 μCi L-[^{3}H]tyrosine ([3,5-^{3}H]-, [2,6-^{3}H]- or [2,3,5,6-^{3}H]-) or 0.1 μCi [7-^{14}C]DA. After 30 min of incubation at 37°C a solution of perchloric acid (PCA) was added to a final concentration of 0.1 *M*. These PCA preparations were stored at −20°C.

Prior to HPLC analysis the preparations were centrifuged at 12 000 *g*, and the supernatant was adjusted to a final pH of 2.2 and perchlorate concentration of 1 *M* [4].

Presentation of results

The mean levels of total DA and DOPAC present in the HPLC fractions collected were calculated from the ED recording. The LSC data directly yielded the fraction contents of the labelled solutes. The ED and LSC measurements observed for the first fraction of each fractionated effluent region were taken as control values and were subtracted from measurements of the further fractions of each region. The rather arbitrary choice of the first collected fraction for this correction sometimes resulted in negative values, when the first fraction was above baseline level. In the diagrams the levels of total DA and DOPAC, as well as those of the labelled compounds, thus corrected, are presented as percentages of the respective top levels observed for each region fractionated.

No correction was made for the lag-time between the moment of electrochemical measurement and that of fractionation. This correction would be very small (the volume from the working electrode of the detector cell to the tube end amounts to ca. 15 μl at most, comprising 11% or less of collected fraction volumes) and at the most would increase the isotope separation observed.

RESULTS

Effect of introduction of one ^{3}H or ^{14}C atom into DA and DOPAC

By incubating homogenates of rat striatal tissue with L-[3,5-^{3}H]tyrosine or [7-^{14}C] DA, preparations containing unlabelled as well as labelled DA and DOPAC were obtained. In both cases the radiolabelled molecules formed contain one radioactive atom per molecule; the second ^{3}H atom from L-[3,5-^{3}H]tyrosine is lost during the enzymic hydroxylation. Perchlorate supernatants of these preparations were analysed by HPLC–ED–LSC as described under Experimental.

Fig. 1 shows the elution profiles noted in a typical experiment with a mixture of ^{3}H- and ^{14}C-labelled preparations. It can be clearly seen that substitution of a tritium for hydrogen in (the phenyl ring of) DA and DOPAC decreased their retention times, whereas introduction of ^{14}C into (the side-chain of) the compounds did not affect their retention. These results were highly reproducible. The

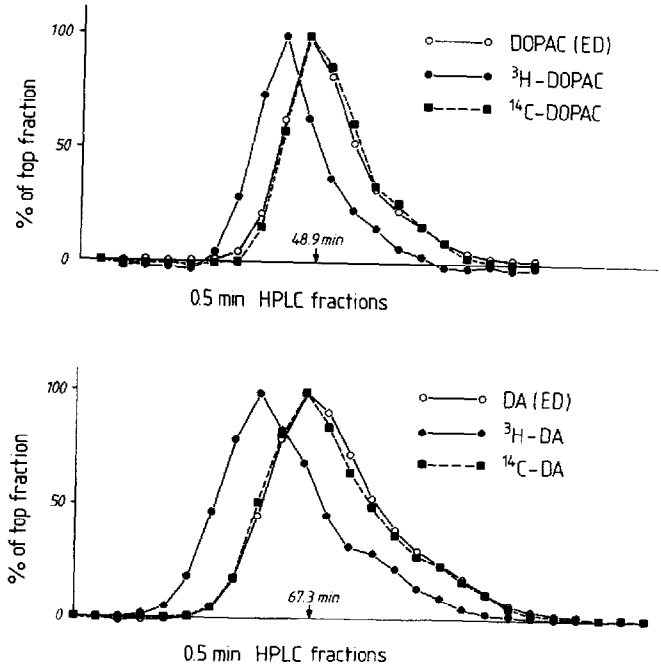

Fig. 1. Isotope fractionation of 5-^{3}H-labelled DOPAC (upper frame) and DA (lower frame) from the respective 7-^{14}C-labelled and unlabelled compounds. The distributions are expressed as a percentage of the respective top levels and plotted against the effluent volume collected. For chromatographic conditions, see Experimental; mobile phase flow-rate, 0.5 ml/min; fraction volume, 0.25 ml. See also Table I, part A.

TABLE I

CHROMATOGRAPHIC PROPERTIES OF TRITIUM-LABELLED AND UNLABELLED DA AND DOPAC

Chromatographic conditions: see Experimental. Mobile phase flow-rate: 0.5 ml/min (A) or 0.4 ml/min (B). Part A: mean ± S.D. of three experiments. Part B: data for unlabelled DA and DOPAC are the mean ± S.D. of the three ED measurements of the separate analyses of single, double and triple tritium-labelled compounds. Capacity factor $k' = (t_R - t_0)/t_0$, where t_R and t_0 are the retention times (at the peak centre), at constant flow-rate, of the solute indicated and of an unretained solute, respectively. Separation factor $\alpha = k'_{\text{unlabelled compound}}/k'_{\text{3H-labelled compound}}$.

Compound	Retention time (min)	Capacity factor (k')	Separation factor (α)
Part A			
DOPAC	48.84 ± 0.41	15.36 ± 0.12	
[5-^{3}H]DOPAC	48.13 ± 0.38	15.17 ± 0.13	1.015 ± 0.001
DA	67.24 ± 0.12	21.14 ± 0.04	
[5-^{3}H]DA	66.19 ± 0.08	20.81 ± 0.03	1.016 ± 0.001
Part B			
DOPAC	64.49 ± 0.11	15.00 ± 0.03	
[5-^{3}H]DOPAC	63.65	14.80	1.015
[2,6-^{3}H]DOPAC	62.97	14.64	1.025
[2,5,6-^{3}H]DOPAC	62.46	14.52	1.031
DA	82.87 ± 0.11	19.27 ± 0.03	
[5-^{3}H]DA	81.51	18.96	1.016
[2,6-^{3}H]DA	80.75	18.78	1.028
[2,5,6-^{3}H]DA	80.18	18.65	1.033

quantitative data of the tritium isotopic fractionation observed in three independent experiments are summarized in Table I (part A). The capacity factor reduction caused by the introduction of a tritium atom is similar for DA and DOPAC, and amounts to ca. 1.5%.

When the chromatographic conditions described under Experimental are applied, tyrosine is scarcely retarded and is present in the solvent front. However, as reported earlier [3], if the parameters of the phase system are changed, an acceptable retention of tyrosine can be brought about, and then tyrosine also appears to show tritium isotope fractionation (results not shown).

Effect of introduction of more ^3H atoms into DA and DOPAC

Rat striatal homogenates were incubated with three different ^3H-labelled L-tyrosine derivatives: 3,5-^3H-, 2,6-^3H- or 2,3,5,6-^3H-labelled. This yielded preparations containing DA and DOPAC molecules labelled with one, two or three ^3H

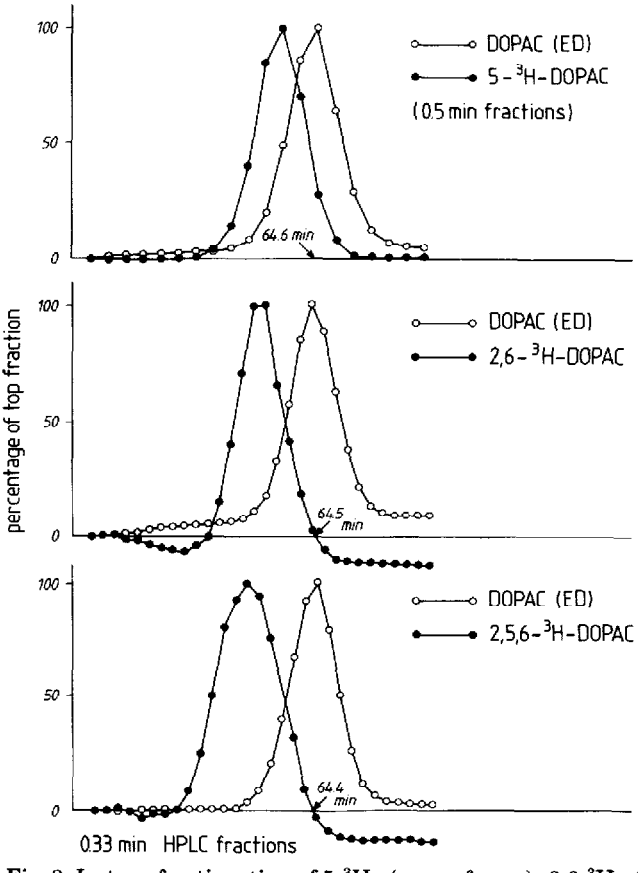

Fig. 2. Isotope fractionation of 5-^3H- (upper frame), 2,6-^3H- (middle frame) and 2,5,6-^3H-labelled (lower frame) DOPAC from unlabelled DOPAC. The distributions are expressed as a percentage of the respective top levels and plotted against the effluent volume collected. For chromatographic conditions, see Experimental; mobile phase flow-rate, 0.4 ml/min; fraction volumes, 0.13 ml (except [5-^3H]DOPAC, 0.20 ml). See also Table I, part B.

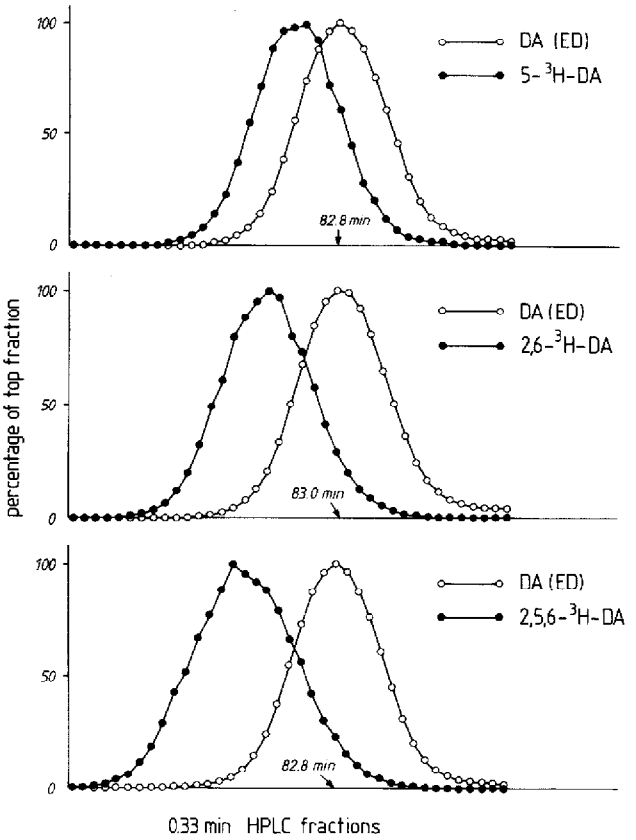

Fig. 3. Isotope fractionation of 5-^{3}H- (upper frame), 2,6-^{3}H- (middle frame) and 2,5,6-^{3}H-labelled (lower frame) DA from unlabelled DA. The distributions are expressed as a percentage of the respective top levels and plotted against the effluent volume collected. For chromatographic conditions, see Experimental; mobile phase flow-rate, 0.4 ml/min; fraction volumes, 0.13 ml. See also Table I, part B.

atoms, respectively, in consequence of the loss of the ^{3}H atom at the C-3 (or C-5) position (see Discussion).

The results of HPLC–ED–LSC analysis of these preparations are shown in Fig. 2 for DOPAC and Fig. 3 for DA; the chromatographic properties of the compounds are summarized in part B of Table I. The presence of more ^{3}H atoms led to a further reduction in the retention time for both compounds: the separation factors for DOPAC amount to 1.015 for one ^{3}H, 1.025 for two ^{3}H and 1.031 for three ^{3}H. The separation factors for DA tend to be somewhat larger: 1.016, 1.028 and 1.033, respectively.

DISCUSSION

For our studies on compartmentation of DA metabolism in rat brain [2], we have developed an HPLC procedure that enables us to analyse relatively large amounts of tissue and to measure both unlabelled and radioactively labelled com-

pounds by combining ED with LSC of the effluent. The phase system selected was a reversed-phase liquid–liquid partition system, using as stationary phase TBP and as mobile phase citrate buffer, containing perchlorate as counter-ion for the amines [3,4].

During routine application of this procedure, it became clear that the chromatographic behaviour of ^{3}H-labelled DA deviated from that of unlabelled DA: for practical reasons, the DA peak elution volume was collected for LSC in two counting vials in two equal portions, on guidance of the ED signal, but the first fraction always was more ^{3}H-enriched than the second. Therefore we decided to perform a detailed fractionation of the HPLC effluent, in order to measure the exact position of the labelled molecules compared with the mass of the compounds.

[^{3}H]DA and [^{3}H]DOPAC were biosynthesized by incubating homogenates of rat striatal tissue with L-[3,5-^{3}H]tyrosine. In these homogenates L-tyrosine is hydroxylated by the enzyme tyrosine hydroxylase into L-dihydroxyphenylalanine (L-DOPA), which in turn is rapidly convcerted into DA by DOPA decarboxylase. DOPAC is formed by enzymic breakdown of DA by monoamine oxidase and aldehyde dehydrogenase [5]. In the tyrosine hydroxylase reaction the hydrogen atom at C-3 (or C-5) is released, so the DA and DOPAC molecules formed from L-[3,5-^{3}H]tyrosine are single labelled at C-5 (or C-3).

HPLC analysis of the preparation thus labelled clearly showed an isotope effect: tritiated molecules are eluted before the unlabelled analogues. The capacity factors of [^{3}H]DA and [^{3}H]DOPAC (at the centre of radioactivity pattern) were ca. 1.5% smaller than those of the mass of the molecules (at the centre of ED registration). In fact the electrochemical detector measures both the unlabelled and the labelled molecules of the compounds. However, as only ca. 0.1% of the molecules are radiolabelled, ED registration can be safely interpreted as representing the unlabelled molecules only. In a modification of the TBP system that is suitable for tyrosine analysis [3] isotope separation was also noted.

To further characterize the isotope fractionation observed, two types of experiment were performed. Firstly, comparison of the effect of ^{3}H and ^{14}C substitution, leading to the same increase in molecular mass, on HPLC behaviour of DA and DOPAC and, secondly, a study of the effect of substitution of more than one tritium atom for hydrogen into the compounds. No separation from the unlabelled compounds was observed for [^{14}C]DA and [^{14}C]DOPAC (formed by incubating striatal homogenate with [7-^{14}C]DA): the ^{14}C distribution pattern and the ED recording exactly coincided, whereas both were clearly different from the ^{3}H distribution profile.

Biosynthesis of DA and DOPAC molecules labelled with two and three tritium atoms was brought about by incubation of striatal homogenates with L-[2,6-^{3}H]tyrosine and L-[2,3,5,6-^{3}H]tyrosine, respectively, two other varieties of tritiated L-tyrosine that are available commercially. In consequence of the enzymic hydroxylation of tyrosine at the C-3 (or C-5) atom, no tritium atom is released from the former substrate and one atom from the latter, resulting in the formation of double and triple tritium-labelled DA and DOPAC, respectively. As to the chromatographic behaviour of the compounds containing one, two or three ^{3}H atoms, a (non-linear) increase in the isotope separation was observed: the effect of three tritium atoms was twice the effect of a single atom.

How can this observed isotope fractionation be accounted for? As stressed, the difference observed between the retention times cannot be due to the lag-time between the electrochemical recording of the peaks and the timepoint of peak collection, because this is less than 3 s and is thus negligible. In addition, the presence of a substantial lag-time would only explain apparent elution of the radioactivity after the recording of the cold compounds by ED, not before. The fact that the ^{3}H-labelled compounds do show isotope fractionation, whereas ^{14}C incorporation does not, excludes a simple increase of the molecular mass as the cause: the 5-^{3}H- and 7-^{14}C-labelled compounds then should show the same change of retention.

A clue may be found in the separation characteristics of the particular phase system used, as observed in earlier experiments [4]. As the retention of amines and acid metabolites appears to be strictly dependent on the counter-ion concentration and the pH of the mobile phase, respectively, separation apparently takes place on the basis of differences in polarity. The shorter retention time of tritium-labelled DA and DOPAC then would point to a higher polarity of these compounds in comparison with their unlabelled counterparts. As far as we know, no direct experimental data for the catecholamines or their metabolites have been published in this respect. But for some other compounds (aromatic and non-aromatic) it has been demonstrated, by examining partition in water–organic solvent systems, that tritium (and deuterium) substitution for hydrogen leads to a decrease of lipophilicity [6,7]. Reasoning further along this line, our results indicate that labelling with ^{14}C at C-7 evidently does not change the polarity of DA and DOPAC. It has to be stressed, however, that the ^{3}H- and ^{14}C-labelled compounds used in our study essentially differ in the respective positions of the isotopic atom in the molecules: tritium was always present in the phenyl ring, whereas the ^{14}C compounds were labelled in the side-chain. We cannot exclude the possibility that the place of incorporation of the isotope also matters for isotope fractionation.

To our knowledge no tritium (or deuterium) isotope effect in HPLC analysis of catecholamines and metabolites in any phase system has been published before. Also, for other types of solute, only a small number of examples of isotope fractionation in HPLC has been reported to date. In general, introduction of tritium tends to increase retention times under normal-phase chromatographic conditions [8,9], whereas retention times are reduced on reversed-phase systems [10,11]. The results described here are in accord with the latter observation. Also, the absence of a ^{14}C isotope effect in our experiments is in line with literature data: one of the groups referred to above, in a reversed-phase HPLC study, used ^{14}C-labelled compounds for comparison and found no isotope fractionation [10].

Recognition of isotope fractionation is extremely important when the non-radioactive form of a compound is used to monitor the elution of the radioactive form from chromatographic systems, or vice versa. In our studies on compartmentation of DA metabolism, measurement of the specific activities of DA and its metabolites is a very important parameter. From the present results it is clear that starting and ending peak collection for LSC on guidance of the onset and

fading of changes of the ED signal, respectively, in our phase system results in the missing of a substantial (and, even more important, probably variable) part of the radioactivity present in a certain component, leading to substantial errors in the specific activity calculations. In the most extreme situation examined, i.e. after incorporation of three tritium atoms in the phenyl ring of DA and DOPAC, about half of the radioactivity has been eluted before a measurable change of the ED signal occurs. An additional problem for closely eluting solutes (e.g. 3-methoxytyramine and DA in the present phase system [4]) is that part of the radioactive content of one component may erroneously be ascribed to another component. The fractionation procedure can therefore only be, and in fact is now, performed by following a strict time schedule [2].

REFERENCES

1 P.D. Klein, Adv. Chromatogr., 3 (1966) 3.
2 F.J.P. Lippens, J.A. van der Krogt, E.L. Noach and C.F.M. van Valkenburg, Neurochem. Int., (1988) in press.
3 C. van Valkenburg, J. van der Krogt, P. Moleman, H. van Berkum, U. Tjaden and J. de Jong, J. Neurosci. Methods, 11 (1984) 29.
4 C. van Valkenburg, U. Tjaden, J. van der Krogt and B. van der Leden, J. Neurochem., 39 (1982) 990.
5 A.S. Horn, J. Korf and B.H.C. Westerink, The Neurobiology of Dopamine, Academic Press, London, 1979.
6 I.M. Kovach and D.M. Quinn, J. Am. Chem. Soc., 105 (1983) 1947.
7 N. El Tayar, H. van de Waterbeemd, M. Gryllaki, B. Testa and W.F. Trager, Int. J. Pharm., 19 (1984) 271.
8 C.N. Filer, R. Fazio and D.G. Ahern, J. Org. Chem., 46 (1981) 3344.
9 B.P. Halloran, D.D. Bikle and J.O. Whitney, J.Chromatogr., 303 (1984) 229.
10 A.M. Jeffrey and P.P. Fu, Anal. Biochem., 77 (1977) 298.
11 J.D. Unadkat and M. Rowland, J. Chromatogr., 261 (1983) 298.